(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(71) Applicants (for all designated States except US): G.IN.E.S.TR.A. S.C. ar.l. [IT/IT]; Via Vicinale Monticino, 1869, I-47020 Diegaro di Cesena (IT). VITRO-PLANT VIVAI DI ZUCCHERELLI GIUSEPPE & C. S.S. [IT/IT]; Via Loreto, 170, I-47023 Cesena (IT).

(72) Inventors; and
(75) Inventors/Applicants (for US only): NAVACCHI, Oriano [IT/IT]; Vitroplant Vivai di Zuccherelli Giuseppe & C.S.S., Via Loreto, 170, I-47023 Cesena (IT). MEZZETTI, Bruno [IT/IT]; Università degli Studi di Ancona, Facoltà di Agraria, Via Ranieri - Monte Dago, I-60100 Ancona (IT). ZUCCHERELLI, Giuseppe [IT/IT]; Vitroplant Vivai di Zuccherelli Giuseppe & C.S.S., Via Loreto, 170, I-47023 Cesena (IT). SPENA, Angelo

[—/IT]; Facoltà di Scienze, Strada Le Grazie, I-37134 Verona (IT).

(74) Agents: CAPASSO, Olga et al.; Ing. Barzanò & Zanardo Roma S.p.A., Via Piemonte, 26, I-00187 Roma (IT).

(54) Title: METHOD TO REGENERATE PLANTS AND USES THEREOF TO MULTIPLY AND/OR TRANSFORM PLANTS

(57) Abstract: A method to produce vegetal cellular bulks with high regeneration capability comprising both a chemical and a mechanical treatment and plant cell bulks obtained therefrom is described. The method of the invention is used advantageously to produce meristematic platforms with high regeneration capability, for the vegetative multiplication of a plant and achievement of a gene transformed plant.

# METHOD TO REGENERATE PLANTS AND USES THEREOF TO MULTIPLY AND/OR TRANSFORM PLANTS

5      The present invention relates to a method for the plant regeneration by induction of adventitious meristems in explants, as well as to applications thereof to multiply and transform plant species.

More particularly the invention relates to a method to regenerate by caulogenesis starting from meristematic bulks, *in vitro* induced in somatic

10     tissue explants, by a breakage of the apical dominance in proliferating shoots and by a hyper-cytokininic stimulus, which determines an hyperplasic and hypertrophic development of the shoot base and then a cellular bulk with a complex cellular and tissue structure. The cellular bulk is characterised by the predominant presence of cells with an high

15     caulogenetic regenerative competence.

The method is applicable for vegetative propagation and multiplication as well as for the genetic manipulation (mediated or direct) of various plant species.

The method allows an efficient regeneration of shoots and therefore

20     of plants, from vegetal tissues. The method can be widely utilised during plant genetic multiplication and transformation.

The regeneration occurs from a "platform" tissue characterised by an high number of cells with high competence for the formation of adventitious meristems (Meristematic Platform). The methodology is of

25     great importance due to the possibility to increase the efficiency and/or to extend both *in vitro* multiplication and genetic transformation techniques to many species of agronomic interest. Therefore the method, increasing the efficiency of multiplication methods, in addition to its use for genetic transformation, allows to implement the application of the vegetal

30     biotechnologies also to plants which are not easily manipulated, also providing cost reduction and standardisation of production techniques.

## Background of the Invention

### *In vitro* culturing techniques and regeneration

The *in vitro* cultures consist of the growth of cells, tissues or organs,

35     isolated from a parent plant on artificial substrates. They are used both for the vegetative propagation and genetic vegetal improvement. They allow a rapid multiplication also in very narrow spaces, starting from minimal amounts of starting material, independently from seasonal cycles. Due to

the potential of the genetic variability generation and selection, in addition to the combination with genetic manipulation techniques, the *in vitro* cultures are an effective tool supporting the conventional techniques for the genetic improvement. These advantages are yet accompanied by drawbacks, as need of specialised workers, higher costs, potential occurrence of undesirable characters in the seedlings and difficulties in the soil adaptation.

The conventional micropropagation technique is based on the proliferation of apical and axillary buds. Direct or indirect differentiation of adventitious buds and/or somatic embryos from de-differentiated tissue allows possible alternatives but, due to the high genetic variability often induced thereby, it results in a difficult practical application in the propagation systems.

Meristematic tissue cultures

Meristematic tissues are undifferentiated tissues causing the growing process of the plant. Meristematic cells are actively self-divided generating both other meristematic and differentiated cells. Meristems can be derived directly from zygote or adult cells regressing to de-differentiated state; they are named "primary" (for example vegetative and root tips) and "secondary" (for example cambium, adventitious meristems and meristemoids) in the first and second case, respectively. The propagation, conventional and *in vitro*, allows to produce, except in the presence of mutations, a clonal population (group of genetically identical individuals)

Propagation Techniques

The more diffused method for the propagation is shoots culture. The propagation occurs by the generation of axillary shoots, isolated and used as explants for subsequent sub-cultures. As primary explants usually lateral 5-10 mm long apexes or buds are used.

The culture of the terminal ends of the meristematic apexes is named meristem culture. This technique is usually used to remove viruses form the plants, often in combination with heat treatment. Explants consists of small portions (0,2-1 mm long) of apical meristems, with one or two leaf primordia.

Nodus culture is a technique derived from shoot culture, more recently provided (Wang, 1977), wherein the stems are localised horizontally on solid substrate.The propagation, like

5    in the shoot culture, occurs by axillary buds.

Major steps of the micropropagation were defined by Murashige (1974). Debergh and Maene (1981) proposed to add 0 stage, comprising the steps before the explant.

Step 0: Selection of the parent plant and preparation.

10    Step 1: Start of the culture.

Step 2: Multiplication.

Step 3: Elongation and induction of the root development.

Step 4: Adaptation.

The passage from step 3 to 4 is critical because the seedlings are

15    adapted to a sterile environment characterised by high humidity, low light intensity and supported by external carbon source (for example sucrose). Therefore it is required to control, after the transplant in a yet sterile soil, the light and humidity conditions until the plants re-acquired the structural and functional autonomy. Adaptation yields depend on the type and quality

20    of the material derived from the previous steps.

Morphogenesis and Differentiation

The generation process of a new organ from a vegetal tissue is named organogenesis or morphogenesis. The organs developing from somatic tissues are named adventitious.

25    In the plants the potential to produce organs or even a complete plant, is owned not only by the meristematic but also by the somatic tissue cells. However not all the plant cells maintain morphogenetic or organogenetic capabilities: the more specialised plant cells, as vascular tissue and sclerenchyma ones, do not modify their differentiated state.

30    De-differentiation and Callogenesis

The cell and tissue transition from a differentiated to undifferentiated state is named de-differentiation or callogenesis and it can be induced in vivo or in vitro following chemical, physical or biological stimuli.

35    Vegetal tissues can growth in an organised way, wherein the cells maintain a well defined structure or in an unorganised way (callus). The organised tissue is constituted of differentiated cells (morphologically and

4

functionally organised), while the unorganised tissue contains a minimal amount of differentiated cells.

Callus is an amorphous tissue generated when cells are divided
5      disorderly. *In vivo* it can be induced in a plant from produced injury, in the presence of microorganism or stress conditions. During the formation of callus, the cellular metabolism passes from a differentiated and quiescent to a meristematic, not specialised and actively dividing state. The *in vitro* induction of callus takes advantage of the same phenomenon which can
10     be amplified or inhibited by the application of chemical and physical factors. The *in vitro* production of callus is increased by carrying out further cuts on explants before their transfer onto induction substrates and by the combination of growth regulators added to the substrate the light and temperature conditions being adjusted. Three are the major factors
15     inducing the callus: 1) explant type, 2) selection of the substrate and culturing conditions and 3) separation of the callus from the explant and culturing maintenance thereof (Constabel, 1984).

Morphogenetic Processes

De-differentiated cells able to generate new organs or plants, as a
20     response to specified stimuli, are named competent. Competent cells can be induced in a determined state wherein they tend to follow a precise, genetically determined, development path; this path can go on cellular differentiation or morphogenesis (Christianson, 1987). The assumption of the determined state can take advantage of the presence of certain growth
25     regulators.

Major morphogenetic processes from somatic tissue, both differentiated and undifferentiated, can lead to the formation of unipolar or bipolar structures. Within the first class are caulogenesis and rhizogenesis, which include the formation of adventitious shoots and roots,
30     respectively; while somatic embryogenesis leads to the formation of somatic embryos having a bipolar development.

Morphogenesis can be direct or indirect, depending on its occurrence in the presence or absence of an undifferentiated proliferating tissue (Hicks, 1980). Most of studied vegetal species is able to generate
35     adventitious roots and buds from explants of various plant tissues and organs, while the formation of adventitious embryos is very less usual.

## Caulogenesis and rhizogenesis

Direct caulogenesis consists of the formation of shoots from somatic tissue, while in the indirect one the shoots are formed from
5    dedifferentiated tissue. In the direct caulogenesis about 48 hours following the transfer of a tissue explant onto the culture substrate initiate mitosis which can originate the meristems of the shoots. In turn meristems can generate primordia, whose disposition appears to be random, tendentiously equidistant.

10   Absence of chimera indicates that the shoots are generated from an epidermis individual cell or few daughter cells, anyway deriving from a single cell (Broertjies and Van Harten, 1978; Broertjes and Keen, 1980). According to other (Norris and Smith, 1981) the beginning of the shoot meristems includes the association and possible inclusion of cells situated
15   below the epidermis. Ability to form adventitious shoots is anyway dependent on plant genotype and often tissue type of the explant too.

Indirect formation of shoots follows a path similar to the direct one: the rapid division of cells leads to the formation of meristematic or meristimoid centers (Torrey, 1966) which can develop in primordia (Bonnet
20   and Torrey, 1966) and successively shoots. Also in this case it is very probable the unicellular origin of the callus structures (Thorpe, 1982), but cells close to the initial one are often induced to the division and incorporation into the new structure (Chlyah, 1974; Smith and Thorpe, 1975).

25   Media promoting the callus development usually do not promote morphogenesis too. However in certain cases, if callus is maintained on the same substrate over long time, organogenesis phenomena are observed. In other cases, in order to induce caulogenesis, it is required the transfer of the callus onto suitable substrates. Cells which will form organs
30   derive from the re-setting of undifferentiated cells or cells maintaining a morphogenetic potential already present in the tissue explant. Except some cases the formation of shoots is promoted in recently isolated tissues and the potential is decreasing with the sub-culture number. Sometime callus lines isolated from the same explant can result in
35   different regenerative responses. This behaviour could result from the fact that primary callus consists of competent and not competent cells or tissues (Street, 1979). Morphogenetic and not morphogenetic callus lines

can maintain their own characteristics over years. Sometimes it is possible, by using particular chemical or physical factors, to induce shoots or roots from apparently not regenerative calli, but often these attempts are unsuccessful. Plants with high regenerative capability and showing low genetic variability can be propagated by adventitious more than axillary shoots.

Callus culture, along with the subsequent seedling regeneration, can be used if the object of the culture is not the propagation but the induction of a new variability to be used for the genetic improvement.

Rhizogenesis is the process by which the roots are formed and, as caulogenesis, can occur directly, from somatic tissue or indirectly through an unorganised step. Firstly rhizogenesis proceeds, till to the primordium induction, in the same manner as caulogenesis. Successively primordia can be differentiated in shoots or roots, as a response to stimuli of different typei (Halperlin, 1069). High levels of auxin usually stimulate the rhizogenesis. Generally in vitro rhizogenesis occurs much more easily than caulogenesis.

Direct caulogenesis often is used for the propagation of various decorative and crop plants. In the past some decorative plants like *Fresia* (Hussey and Hargraves, 1974) and *Pelargonium* (Holdgate, 1977) were propagated by indirect formation of adventitious shoots. However currently it is generally consolidated that this process involves a too high variability to maintain the commercial characteristics of the plant.

Adventitious roots and shoots can associated to produce a plant according to different techniques: 1) the caulogenesis is followed by formation of adventitious roots (Skoog, 1944); 2) the adventitious buds are produced on adventitious roots (Earle and Torrey, 1965); 3) the independent regeneration of roots and buds is followed by their integration in a single axis (Steward *et al.*, 1958; Pilet, 1961; Kato and Takeuchi, 1963).

Somatic Embryogenesis

Somatic embryogenesis can occur directly or indirectly. *In vitro* direct embryogenesis consists of the formation of somatic embryos on tissue explants without involvement of undifferentiated tissue. *In vitro* direct embryogenesis principally was described in gametophyte and sporophyte tissues, in association with gametophyte or derived from

fertilisation thereof (Smith and Krikorian). Practically it occurs only in cells suitable to produce embryos, named pre-embryogenically determined cells (PEDC) and the transfer onto the culture medium has only the function to
5   increase such a process (Sharp *et al.*, 1980; Evans *et al.*, 1981a and Sharp and Evans, 1982).

Female gametophyte of some species, as certain *Citrus* species, is able to generate naturally adventitious somatic embryos. This tendency to have in the same gametophyte more than one embryo, namely one
10  zygotic and several adventitious, is named polyembryony.

Somatic embryos can be formed indirectly when undifferentiated callus cells are induced to the embryogenetic determination (Sharp *et al.*, 1980). These cells are named "induced embryogenetic determined cells" (IEDC). Although the embryos are difficult to be observed, they can be
15  distinguished from adventitious shoots in that, differently from these, they are bipolar, comprising both an apical and a radical pole, an axis of the shoot and cotyledons, do not have vascular connections with adjacent parental tissues. Embryos (both zygotic and somatic) are in fact new individuals deriving from single cells and not having vascular connections
20  with parental tissues (Haccius, 1978).

Somatic embryos can develop from a single cell, directly from somatic tissue, on callus or cellular suspensions (Backs-Husemann and Reinert, 1970; Nomura and Komamine, 1986a; Miura and Tabata, 1986) and from protoplasts (Miura and Tabata, 1986). Embryogenetically
25  determined cell can be subjected to a periclinal division forming a cytoplasm rich terminal cell, which will generate embryo, and a large and vacuolated basal cell which in turn can divide a few times generating a suspensor. If the basal cell divides according to anticlinal planes or randomly, can generate pro-embryonal cellular complexes or anomalous
30  embryos with multi-serial suspensor (Trigiano *et al.*, 1989).

In the indirect embryogenesis most of the regenerated plants is genetically normal, higher variability being found in somatic embryos induced in callus cultures or suspension following a long period under not optimal growth conditions (Orton, 1985). Propagation by indirect somatic
35  embryogenesis is applied to few species, usually monocotyledons like cereals and palms, wherein the propagation by the shoots culture or caulogenesis is ineffective. In some cases direct embryogenesis was

8

effectively used for the micropropagation, as for *Helianthus annuus* L. (Pelissier *et al.,* 1990) and *Juglans regia* L.(McGranahan *et al.,* 1988b).

Possibility to obtain effective differentiation protocols for the various
5     iinteresting species is usually determined by the study and acknowledgement of the genotype response (plant, cell, tissue) to various factors possibly affecting the regulation of the various steps of the morphogenetic process (induction, determination and development).

Usually in this process are involved the following factors:
10    — growth regulators (phytohormones, stress inductors, oligosaccharides, etc.);
      — physical factors (light and temperature);
      — regeneration methods.

Within this scope therefore studies about the relationship among
15    these factors are particularly important in order to define effective methods for the regeneration of plants, which are also characterised by high genetic stability, mainly when the application relates to the multiplication or genetic manipulation thereof.

Variability of the morphogenetic processes
20    Plants regenerated by embryogenesis are usually genetically homogenous, while in some cases, when regeneration by caulogenesis or rhizogenesis is used, it is possible to produce chimeras. The reason of such a behaviour is based on the fact that the adventitious organs can likely have a multicellular origin (Norris and Smith, 1981), while the
25    embryos usually have a unicellular origin.

From above it is apparent the advantage of providing vegetative propagation systems based on the production of cells with high regenerative competence without a long induction period and de-differentiating cultures as a guarantee of high genetic stability. It is further
30    advantageous the insertion of genes in individual cells and the production by regeneration of homogeneously transformed plants for the expression of one or more heterologous genes.

Genetic Transformation

Transformation is a process by which exogenous DNA is introduced
35    into a vegetal cell. In the middle eighty first transgenic plant was produced

from *Nicotiana plumbaginifolia* by the *Agrobacterium* mediated technique (Horsch *et al.*, 1984; DeBlock *et al.*, 1984). Thereafter a great number of species was engineered using different methodologies (Gasser and

5    Fraley, 1989). One of such techniques uses the transformation of vegetal cells from a tissue explant (protoplasts). The production of transgenic plants depends essentially on the introduction frequency of the interest gene and the ability of the transformed cells to regenerate the whole plant, shoots or roots. Both the aspects can represent limiting factors for the fruit

10   plants.

Evidence of the occurred transformation is represented by the expression of introduced DNA in the vegetal cells: for this reason into the transforming vector also a marker gene is inserted which, when expressed, provides the plant with a characteristic phenotype.

15   To obtain transgenic plants it is required: a) to isolate the interest gene; b) to have a vector system allowing the insertion thereof into the plant; c) to have a good transformation technique; d) to have a good regeneration technique; e) to have a good selection technique.

Genetic transformation techniques

20   · Major transformation techniques can be indirect, when they take advantage of the intrinsic ability of the pathogenic bacterial and viral agents to transfer genes, or direct, wherein exogenous DNA is directly inserted into the cell, without any interaction with pathogenic agent.

Indirect methods use microbial expression vectors. Gene transfer

25   into the vegetal cell is facilitated by the naturally occrring gene transfer system of *Agrobacterium tumefaciens* (Gheysen *et al.*, 1985; Fraley *et al.*, 1986; Lichtenstein and Fuller, 1987; Zambryski *et al.*, 1989; Schuerman and Dandekar, 1991). Also DNA (Gronenborg and Matzeit, 1989) and RNA (French *et al.*, 1986) viruses were modified in order to insert an

30   exogenous gene replacing part of the viral genome, thus generating a viral defective particle able to carry out gene transfer.

The *Agrobacterium* mediated transformation technique was the first tool for the transformation of many dicotyledonae. It is certainly preferred method for all the vegetal species for which T-DNA transfer is possible due

35   to relative transfer easiness and precision in undamaged and able to regenerate explants explants.

However many vegetal species, among which economically important cereals like rice, maize and wheat, are not easily transformed by conventional indirect method. Therefore alternative systems were provided

5   wherein DNA insertion into the host cell occurs as free DNA followed by the random integration into the genome. Among these the biolistic method represent a mechanical method for the introduction of DNA into most of the vegetal species. It is advantageous in all the situations wherein the Agrobacterium mediated transformation and free transfer into protoplasts

10  are not possible. The technique is based on the acceleration of heavy particles with some micrometer diameter, principally gold or tungsten, which can penetrate through the cellular wall and plasmatic membrane of undamaged vegetal cells, thus delivering the genetic matter. Because the small holes through the wall and plasmalemma quickly self-close, the

15  produced injuries are temporary and do not compromise irreversibly the cell integrity.

The first transgenic plant, simultaneously obtained by Agrobacterium (Hincee et al., 1988) and biolistic method (McCabe et al., 1988), was soy-bean. The most interesting result was the production of

20  fertile transgenic maize plants (Fromm et al., 1990; Gordon-Kamm et al., 1990).

Selection Systems

Many genes encoding for antibiotics or herbicides can be used as selection markers, usually required for an efficient production of transgenic

25  plants. Selection agents are different each other relating to the toxicity grade against the plant, which depends on the size and development step of the cell o tissues. In the presence of the selection agent indifferentiated cells respond differently to the explants of organs as leaves and cotyledons. In addition the susceptibility to the various agents depends on

30  the species. Therefore for each transformation and regeneration protocol it is required to carry out the precise determination of the concentration of the selection agent which allows on the hand the growth and development of the transformed cells and on the other hand inhibits the proliferation of undifferentiated cells. Often herbicides are more toxic than antibiotics

35  against vegetal tissues such that their use is carried out only successively, i.e. when it is certain that the transformed cells produce enzymes in such amount to induce resistance (Donn et al., 1990).

For many herbaceous species, usually propagated by gametes, are provided effective efficacious protocols for regeneration, also by somatic embryogenesis, which afford effective multiplication and transformation results. For some of herbaceous and mainly many of the arboreal species there are yet remarkable difficulties in developing efficacious regeneration protocols suitable for genetic multiplication and transformation. In many cases there are specific problems also for the application of standard transformation protocols.

The method of the invention aims to overcome these problems, by providing an efficacious genetic transformation protocol also for plants proving recalcitrant to the conventional techniques.

Prior art techniques prove to have an effective application only for species having a cellular structure of the various somatic tissues characterised by high cellular competence for organogenesis and somatic embryogenesis processes. Currently known and/or used regeneration protocols depend on the cellular competence of the interest plant genotype (Genus-Species-Variety) and tissue type (both meristematic and somatic: leaves, stem, stipule, etc.). Many vegetal species having high agronomic interest show an extremely reduced competence to the regeneration of their various cellular typologies. This behaviour, mainly apparent in many woody species, often represents the determining factor in limiting the application of the *in vitro* multiplication technologies and gene manipulation, due to reduced efficiency and difficulty in obtaining the regeneration of interest gene modified plant, respectively.

The method of the invention allows to obtain the formation of tissues characterised by a major cellular component having high competence for the regeneration of adventitious buds. For example, the method allows to rationalise the production process of nurseries because it can be used both for the multiplication and transformation. Thus the costs for the production and implementation of genetic vegetal biotechnological innovations are reduced.

Description of the Invention

The present invention consists of method of *in vitro* culturing plant tissues that, by inducing and culturing meristematic cellular bulks, allows to produce cellular layers (platforms) characterised by an high regeneration efficiency, via caulogenesis, of new plants.

The basic principle of the method is the induction of growing cellular bulks with high regenerative competence from which, via sectioning, it is possible to obtain cellular layers (platforms), again with high regenerative competence. The choice of the initial concentration of the growth regulators is defined on the base of the indications from individual species during the *in vitro* standard proliferation step (proliferation of axillary buds).

Availability of *in vitro* proliferating shoots and knowledge of growth regulators (particularly cytokinins) combinations required for the standard proliferation of axillary buds prove to be essential for the induction and production of meristematic bulks). Therefore culture substrates characterised by higher cytokinin concentrations were provided, thereby inducing higher *in vitro* proliferation of axillary buds, which results in the generation of dense cellular bulks consisting of different type meristematic tissues. This evolution usually is reached during 3-4 sequential sub-cultures wherein the initial concentration of cytokinins in the substrate gradually is triplicated. In parallel to this hormonal treatment at each culture all the more developed bud apexes are removed (topping) not touching the base. Therefore the method of the invention to produce meristematic bulks is based on the combined effect of the chemical (hormonal) and mechanical (breakage of the apical dominance: topping) treatment.

The chemical treatment consists of progressive increase of the cytokininis content in the substrate of the proliferating shoots, reaching in few sub-cultures (3-5) an effective concentration of the cytikininis at least three times higher than initial value in the proliferating substrate (1-3).

The mechanical treatment includes, at each transplant of the proliferating shoots, without removing the base, the removal of the apex of the individual shoots (topping) originating from axillary buds. This treatment promotes a strong growth of the base which more and more is characterised by an increasing presence of various meristematic centres.

Thus the not removed base of the shoots becomes more and more developed and hypertrophic till to the formation of a bulk characterised by an parenchymatic base with cells having high competence for the differentiation of adventitious buds. Histologically these bulks are characterised by a more internal part of dense tissue, made of the shoot base co-growth with the buds. Cross-sections of the bulks show the

simultaneous presence of primary and secondary meristems and pro-
meristematic primordia immersed in highly vascularized parenchyma.
Particularly it is apparent high formation, also in very limited tissue
portions, of primary meristems at different evolution steps: from

parenchymatic cells (callus) to complete primary meristem (adventitious
bud) comprising all the intermediates steps (nodules, meristematic
primordium). These cellular bulks therefore prove to be with high
regeneration competence which can be maintained during successive
sub-culture on substrates characterised by the cytokininis concentrations
reached in the last step of the bulk induction. The essential characteristic
of the produced bulks is that from their fragmentation, following three or
four culturing weeks, identical formations are obtained. In fact these
meristematic bulks can be maintained in culture and multiplied by
sectioning (about 2 mm thick homogenous sections) resulting in new
meristematic platforms characterised by the same regenerative efficiency.

This "meristematic bulk-platform" cycle prove to be constant for the
various analysed species and usable both for the vegetative multiplication
(cloning) and genetic transfer.

Application 1 – Vegetative Multiplication

Effective induction and maintenance of meristematic bulks (MB)
and high regenerative efficiency of new adventitious shoots of
meristematic platforms represent an effective multiplication method
resulting in simplified techniques for the in vitro manipulation of the vegetal
matter (simple sectioning of the bulks) and the achievement of explants
maintaining yet high regenerative efficiency.

The method is suitable to produce large amounts of these cellular
bulks (both on liquid and solid substrates), easily to be sectioned and
usable for the production and differentiation of new shoots and plants in
great number. Thus formed meristematic bulks can be used for the
production, via regular sectioning, of cellular platforms (about 2 cm thick
and 1 cm long and wide sections) which maintain the characteristic of
meristematic-vascularized bulks with high competence to determine the
regeneration of new adventitious shoots (caulogenesis).

The application of the method for the vegetal propagation is
demonstrated by effective and stable proliferation of the bulks (via

regeneration) and effective regeneration obtained from cellular platforms by mechanical fragmentation thereof. High efficiency of this technique can be further increased by potential automation-robotization of some steps 5    (bioreactor-grinder).

Application 2 – Genetic Transformation
Cellular platforms obtained by the method of the invention are advantageously applied for the genetic transfer both by *Agrobacterium* 10    *tumefaciens* mediation and direct techniques. In this case the combined effect of the injury and high frequency of regeneration competent cells promote high efficiency also for the transformation.

Characteristics of the cells constituting meristematic bulks and sectioning thereof, carried out during the regeneration-multiplication step, 15    assist to create optimum conditions to promote genetic transformation events. Already formed meristems, not competent for the transformation, develop shoots which will be removed over a few selection cycles

Particularly experiments using *Agrobacterium tumefaciens* EHA 105 strain containing pTi EHA105 unarmed plasmid (Hood *et al.*, 1993) and 20    various vectors, particularly the binary vector containing neomycin photo transferase marker gene (nptII), which confers kanamycin resistance, under the control of nopaline synthetase promoter (Pnos), are carried out.

First operation to be carried out in the preparation of the transformation explants is the removal of all the outer surface portion, 25    exposing inner tissue till to the formation of about 1 cm small cube made of clear and dense tissue. Following the small cube is sectioned forming cellular platforms showing high regenerative efficiency and large injury surface, considered necessary to promote the efficient transformation. In fact, mainly referring to *Agrobacterium* mediated transformation, the 30    infection and transformation capability is affected by the activity of the compounds released from the surface of the injured cells during the preparation of the sections.

For the growth and infection of the bacterial strains standard techniques are used.

35    The infection, further to growth of bacteria, is carried out according to the conventional PM infection method, preferably including also a treatment with acetosyringone and syringaldehyde (25 $\mu$l), which are

organic compounds known to activate the expression of *vir* genes and, therefore, to mobilise the DNA of the binary plasmid.

At the end of infection the explants are dehydrated using 3MM sterile absorbent paper and transferred into Petri dishes containing the regeneration substrate characterised by hormonal concentration as defined for the PM regeneration. Anyway the transformation occurs for an extremely reduced number of cells and the following selection step is extremely important to discriminate stable and homogeneous transformation events. To this object a protocol of continuous regeneration in selecting medium, based on various passages of PM on substrates added with same concentrations of growth regulators and increasing doses of the selecting factor (kanamycin), from a minimum level of 25 mg/l to a final concentration (after 4 sub-cultures) from 50 to 100 mg/l, depending on the kanamycin sensitivity of the species, is started.

First shoots developing from PM usually originate from already formed meristems, therefore probably without stabile transgenic origin. However it is extremely important to remove no part of tissue or shoots during the initial phase of the selection, in order to maintain also new partially modified shoots (transgenic chimeras). Very often in these cases only few cells of the meristem or section thereof are transformed. In this way chimeric individuals are formed. By maintaining the selective pressure of kanamycin the probability of isolating the chimera with subsequent advantage for transgenic cells increases. In fact also for in vitro cultures it is difficult to equilibrate the chimeras which usually result in an advantage to one of two genotypes.

This is explained by a different response to the in vitro culture conditions of two genotypes. With a transgenic-not transgenic chimera the selective pressure exerted by kanamycin results in selective advantage of transgenic cells.

A moderate selection during proliferation and subsequent radication with kanamycin (in radication it is possible to obtain a better selection) are preferred for easy rooting species. It is extremely important to proceed by a continuous regeneration over quite long periods and frequent sub-cultures. Continuous regeneration means to maintain, after the first regeneration, continuously the explants in regeneration and selection substrate. In fact it is believed that the initial regeneration events usually

are related to already formed and therefore rarely transgenic meristems. In a following step, the vegetal matter being maintained in a regeneration-selection substrate, the probability to increase the frequency of

5     transformed cells, whose subsequent differentiation promotes the regeneration and development of permanently and homogeneously genetically modified individuals, is higher.

Continuous selective regeneration from PM obtained regenerations results in an amplification of differentiated tissues with genetically

10    manipulated cells and consequent stabilisation of chimeras and increase of the transgenic frequency. To verify the transformation homogeneity of isolated shoots a rooting final step is carried out again in the presence of kanamycin. In fact the generation of roots in the presence of kanamycin corresponds, very probably, to an occurred transformation.

15    The present invention will be described below by way of specific embodiments to be considered illustrative but not limiting, wherein reference will be made to the following figures:

Figure 1. Southern Blot analysis on 0,7 % agarose gel in 0,5 X TBE of DNA of *Defh9-iaaM* transgenic, vine, cv "Thompson". Column 1, HindIII

20    digested clone 4 DNA. Column 2, HindIII/EcoRI digested clone 4 DNA.

Figure 2. Southern Blot analysis of DNA of two *DefH9-iaaM* transgenic, vine, cv Silcora (I.G. 235023). Column 1, HindIII digested clone 29 DNA. Column 2, HindIII/EcoRI digested clone 29 DNA. Column 3, HindIII digested clone 35 DNA. Column 4, HindIII/EcoRI digested clone

25    35 DNA. Column 5, HindIII digested control DNA. Column 6, HindIII/EcoRI digested control DNA.

Vegetative Multiplication

The described method for the regeneration by the formation of a "platform" tissue characterised by very numerous cells with high

30    competence state for the formation of adventitious meristems (Meristematic Platform) has been tested and developed for a great number of species of agronomic and forest interest.

Description of the method applicable for dessert grapes (*Vitis vinifera*; cvs Thompson Seedless and Silcora (I.G. 235023)

35    Meristematic bulks induced using proliferating shoots, are *in vitro* stabilised in 500 cc "Fidenza" pots in A substrate containing: macroelements listed in Table 1; microelements and vitamins according to

Murashige and Skoog (1962); and 0,8 mg/l 6-Benzyl adenine hormone; 3 % sucrose; 0,7 % Agar.

5

## Table 1

| Macroelements | (mg/l) |
|---|---|
| $KNO_3$ | 1050 |
| $NH_4NO_3$ | 400 |
| $KH_2PO_4$ | 200 |
| $MgSO_4\ 7H_2O$ | 400 |
| $CaNO_3$ | 750 |
| $NaH_2PO_4$ | 200 |

10

Meristematic bulks are subjected, at each sub-culture, to a severe topping to stimulate a more and more increasing proliferation at the base
15    of the cluster, according to the scheme showed in Table 2.

## Table 2

| First sub-culture | Second Sub-culture | Third sub-culture | Fourth sub-culture |
|---|---|---|---|
| Severe topping | Severe topping | Severe topping | Production and |
| without removal | without removal | without removal | maintenance of |
| of the cluster base | of the cluster base | of the cluster base | Meristematic Bulks |
| Day 0 | Day 30 | Day 60 | Day 90 |
| BAP 1 mg/l | BAP 2 mg/l | BAP 3 mg/l | BAP 3 mg/l |
| NAA 0,01 mg/l | NAA 0,01 mg/l | NAA 0,01 mg/l | NAA 0,01 mg/l |

20

25

Shoots are stimulated at the same time with hormonal and mechanical stimuli together with severe topping. In this way the shoots become more and more large and hypertrophic, till to obtain bulks wherein buds and shoots are co-growth in a dense and callus and meristematic
30    primordia rich bulk. Histologically the bulks are characterised by more internal part of dense tissue consisting of the shoot base co-growth with the buds. In the cross-section many bulks of primary and secondary meristems and pro-meristematic primordia immersed in a richly vascularised parenchyma are apparent.
35    Meristematic bulks easily can be propagated by manual or mechanical fragmentation. From these further bulks on the same substrate, containing 3 mg/l BAP for the induction, can be obtained.

By removing the external part of the bulks, rich of already formed meristems and buds, sections, of about of 1 cm$^2$ surface and 2 mm thickness, can be prepared to constitute MPs usable for the multiplication

5   and transformation.

Results of the regeneration by multiplication process

The described method allows to produce the meristematic platforms with high regeneration capability, resulting in higher production efficiency of shoots than proliferating rate usually found during a conventional *in vitro*

10  proliferation step from axillary buds and also than regeneration rate from somatic tissue (usually leaf and stalk).

In Table 3 the proliferation rates obtained on overage for the two vine varieties by applying the PM regeneration and conventional proliferation and regeneration methods from axillary buds and somatic

15  tissues, respectively, are compared.

### Table 3

| Proliferation rate from axillary buds | Regeneration rate from foliar or other somatic tissues | PM regeneration rate |
|---|---|---|
| 3-5 | 0,1-0,5 | 20-30 |

20

Use of the method for other agronomically interesting species

Analogously the method is used for a great number for other species, for which was proved the possibility of obtaining the formation of

25  the meristematic bulk. Particularly good results of the PM regeneration efficiency for the species listed in Table 4 were obtained.

### Table 4

| Temperate fruit | Sub-tropical fruit | Horticultural | Officinal forest shrubby |
|---|---|---|---|
| Peach | Almond | Artichoke | Eucaliptus |
| Pear | Orange | Been | Walnut |
| Apple | | | |
| Kiwi | | | |
| Strawberry | | | |

35

In Table 5 the proliferation rates obtained for the various tested species by applying the PM regeneration and conventional proliferation

and regeneration methods from axillary buds and somatic tissues, respectively, are compared.

Table 5

| Species | Proliferation rate from axillary buds | Proliferation rate from foliar or other somatic tissues | PM regeneration rate |
|---|---|---|---|
| Peach | 2-4 | 0,01-0,1 | 20-30 |
| Pear | 2-4 | 0,01-0,5 | 20-30 |
| Apple | 3-5 | 0,1-0,8 | 20-30 |
| Kiwi | 2-3 | 0,1-0,8 | 10-15 |
| Strawberry | 5-8 | 0,1-0,8 | 15-25 |
| Almond | 3-5 | 0,01-0,1 | 20-25 |
| Orange | 3-5 | 0,01-0,1 | 10-15 |
| Artichoke | 3-5 | 0-0,0,1 | 20-25 |
| Bean | 3-5 | 0-0,01 | 15-20 |
| Eucaliptus | 4-6 | 0,01-0,1 | 20-30 |
| Walnut | 2-4 | 0-0,2 | 20-30 |

Gene Transfer

Using the described PM regeneration, Agrobacterium mediated gene transfer and selection methods, genetically modified shoots were obtained with very high frequency (see Table 6 below).

Description of the method for the vine

Meristematic platforms, after the infection and co-culturing period with *Agrobacterium*, were placed on regeneration substrate consisting of same substrate as used in the third sub-culture during the induction step added with BAP 3 mg/l, cefotaxime 50 mg/l and kanamycin 50 mg/l. This step is important because the transformation occurs for a reduced number of cells and the regeneration, under the selective pressure of kanamycin, gives a selection advantage to the cells and transgenic shoots at each regeneration cycle.

The procedure, repeated for various regeneration cycles, results in the amplification of the transformation events producing a redundancy of transgenic individual copies, but also in increase of the chimera stabilisation and transgenic frequency. The process involves the presence of "escapes" in great number, i.e. apparently transgenic shoots which

actually avoided casually the selection agent effect, requiring therefore a severe final selection.

All the matter is placed to radication on a substrate with these 5 components: macroelements (Quoirin and Lepoivre medium, 1977); microelements and vitamins (Murashige and Skoog medium, 1962); Hormones: IBA 1 mg/land IAA 1 mg/l; sucrose 3 %; agar 0,7 %, kanamycin 50 mg/l.

<u>Molecular analysis</u>

10 The confirmation of transegenic origin of kanamycin rooted shoots is carried out for two vine varieties by Southern Blot analysis.

Figure 1 shows Southern Blot analysis of *Defh9-iaaM* transgene transgenic cv "Thompson" vine DNA. The probe used, homologous to nptII gene, is of 544 bp, produced by PCR using following primers:

15 5'CAGAGTCCCGCTCAGAAGAACTCGTCA3'    and

5'GGAAGGGACTGGCTGCTATTGGGCGAA3'

The plant contains 4 copies of T-DNA inserted into the genoma. Only one of four fragments, about 8,6, 7,5 and 2,8 kb, is cut by EcoRI, as deduced from the disappearance of the heaviest fragment by double 20 digestion and concurrent detection of 2,4 Kb fragment. Because the distance from HindIII site, present in T-DNA at nptII gene 5', to T-DNA right border (RB) is about 2 kb, EcoRI site is present in genomic DNA of the vine transgenic plant at 400 bp from the insertion site.

Figure 2 shows Southern Blot analysis of *Defh9-iaaM* transgene 25 transgenic cv Silcora (I.G. 235023) two vine DNA. The probe used, homologous to *iaaM* gene encoding region, is of 589 bp, produced by PCR using following primers:

5'AACAAGCTTCCCACCACCATCCAG3'    and

5'GCATGCTCTTTTCACCCGTATTAG3'

30 Clone 29 contains 3 copies of T-DNA inserted into the genoma, as deduced from the presence of three bands of about 1,2, 3,5 and 6 kb, produced by HindIII enzyme digestion and homologous to the probe obtained from *Defh9-iaaM* gene promoter. 35 plant contains only one T-DNA copy, as deduced from the detection of a single band of about 2,1 35 Kb. In both cases the HinfIII and EcoRI double digestion produce an about 0,8 Kb band.

Table 6

Stable transformation events from 100 *Agrobacterium* infected explants

| Species | Regeneration from leaf | Somatic embryogenesis | PM regeneration |
|---|---|---|---|
| Vine | 0 | 2 | 10 |

1.

Above described regeneration method already prove to be very efficient, but yet can allow to reach further advantages in the development of new vegetative propagation techniques when adjusted for automated culturing systems (bioreactors). The fragmentation of meristematic bulks results in a continuos tissue growth associated with new meristems differentiation.

BIBLIOGRAPHY

— Backs-Husemann D., Reinert J. (1970, Protoplasma 70:49-60).
— Bonnet H.T., Torrey J.C. (1966). Am. J. Bot. 53:496-507.
— Broertjes C., Keen A., (1980), Euphytica 29:73-83.
— Broertjies C., Van Harten A.M., (1978). Application of mutation breeding in the improvement of vegetatively propagated crops. Elsevier Scientific Publishing Co. Amsterdam, Oxford, New York.
— Chlyah H., (1974). Plant Physiol. 54:341-348.
— Christianson M.L. (1987). Casual effects in morphogenesis. 45-46 in Green *et al.*(ed.), 1987.
— Constabel F., (1984), Callus culture: induction and maintenance. In Vasil I.K. (Ed.) Cell Culture and Somatic Cell Genetic of Plants. Vol I 27-35. Academic Press Inc. Orlando, Florida.
— Debergh P.C. and Maene L.J. (1981). Sciencia Hort. 14:335-345.
— De Block M. *et al.*(1984). EMBO J. 3:1681-1689.
— Donn G., Nilges M. and Morocz S., (1990). Stable transformation of maize with a chimaeric modified phosphinothricin-acetyltransferase gene from *Streptomyces virido-chromogenes*. 7th Intl. Congress of Plant Tissue and Cell Culture. Abstracts No A2-38, p. 63.
— Earle E.D., Torrey J.G., (1965). Am. J. Botany. 52:891-899.
— Evans D.A., Sharp W.R., Flick C.E., 1981. Growth and behaviour of cell cultures: embryogenesis and organogenesis. Pp 45-113 in Thorpe T.A. (ed.) 1981. Plant Tissue Culture. Methods and Applications in Agriculture. Academic Press, New York, London, Toronto.

- Fraley R.T., Rigers S.G., Horsh R.B. (1986). CRC Reviews in Plant Science 4:1-46.
- French R., Janda M., Ahlquist P. (1986), Science 231:1294-1297.
- Fromm M.E. et al., (1990). Bio/Techology 8:833-839.
- Fraley R.T. (1989). Science 244:1293-1299.
- Gheysen G. *et al.*(1985). DNA flux across genetic barriers: the crown gall phenomenon. In: Hohn B., Dennis E.S. (Ed.). Plant Gene Resource: Genetic Flux in plants. Pp. 11-47. Springer-Verlag, New York.
- Gordon-Kamm W.J., et al (1990). Plant Cell 2:603-618.
- Gronenborg B., Matzeit V. (1989). Plant gene vectors and genetic transformation: plant viruses as vectors. In: Schell J. Vasil J.K. (Ed.) Cell Culture and Somatic Cell Genetics of Plants. Vol 6. Pp. 69-100. Academic Press, New York.
- Haccius B., (1978). Phytomorph. 28:74-81.
- Halperlin W. (1969). Ann. Rev. Plant Physiol. 20:395-418.
- Hicks G.S. 1980. Bot. Rev. 46: 1-23.
- Hinchee M.A.W. *et al.*(1988). Production of transgenic soybean plants using *Agrobacterium*-mediated DNA Transfer. Bio/Technology 6:915-922.
- Holdgate D.P. 1977: Propagation of ornamental by tissue culture. Pp 18-43 in Reinert and Bajaj (Eds.) 1977.
- Hood E.E., et al., (1993). Transgenic Research 2:208-218.
- Horsch R.B. et al., (1984). Science 223:496-498.
- Hussey G., Hargreaves J. 1974. Ann. Rep. John Innes Inst. Pp. 58-59.
- Kato H., Takeuchi M., 1963. Plant Cell Physiol. 4:453-463.
- Lichtenstein C.P., Fuller S.L. (1987). Genetic Engineering 6:103-183.
- McCabe D.E., et al. (1988). Bio/Technology 6:923-926.
- McGranahan G.H., et al., (1988). Bio/Technology 6:800-804.
- Miura Y., Tabata M. (1986). Plant Cell Rep. 5:310-313.
- Murashige T. (1974). Ann. Rev. Plant Physiol. 25:135-166.
- Murashige T., Skoog F., (1962). Physiol. Plant. 15:473-497.
- Nomura K., Komamine A. (1986). New Phytol. 104:25-32.
- Norris R.E., Smith R.H. (1981). Plant Physiol.. 67:117 (Abst. 661).
- Orton T.J. (1985). Plant Cell. Tiss. Organ Cult. 4:159-169.
- Pelissier B., et al. (1990). Plant Cell Rep. 9:47-50.

- Pilet P.E. (1961). Ber. Schweitz. Botan. Ges. 71, 189-208.
- Quoirin M., Lepoivre P. (1977). Acta Hort. 78:437-442.
- Schuerman P., Dandekar A.M. (1991). Potentials of woody plant transformation. In: Biswas B.B., Harris J.R. (ed.). Plant Genetic Engineering. Subcellular Biochemistry. Vol. 19, pp. 81-105. Plenum Press, New York.
- Sharp W.R., Evans D.A., 1982. Application of somatic embryogenesis to crop improvement. In: Fujiwara A. (Ed.) Plant Tissue Culture. 1982. Proc. 5th Int. Cong. Plant Tiss. Cell Cult. Japan. Jap. Assoc. Plant Tissue Culture; Tokyo, pp. 759-762.
- Sharp W.R. et al., (1980). Hort. Rev. 2:268-310.
- Skoog F., (1944). Amer. J. Botany 31:19-24.
- Smith D.L., Thorpe T.A. (1975). J. Exp. Bot. 26:184-192.
- Smith D.L., Krikorian A.D., (1988). Am. J. Bot. 58:103-110.
- Steward R.N., Maspes M.O., Mears K., (1958). Brookhaven Symp. Biol. 16:73-88.
- Street H.E. (1979): Embryogenesis and chemically induced organogenesis. Pp. 123-153 in Sharp W.R., Larsen P.O., Paddock E.F. and Raghavan V. (Eds.) 1979. Ohio State Univ. Press, Columbus.
- Thorpe T.A. (1982). Callus organisation and *de novo* formation of shoosts and embryos *in vitro*. In: Tomes D.T., Ellis B.E., Harney P.M., Kasha K.J., Peterson R.L. (Ed.). Application of Plant Cell and Tissue Culture to Agriculture and Industry. Pp. 115-138. Guelph: University of Guelph.
- Torrey J.G. (1966). Adv. Morphogen. 5:39-91.
- Trigiano R.N., et al. (1989). Bot. Gaz. 150:72-77.
- Wang P.J. (1977). Regeneration of virus-free potato from tissue culture. Pp. 386-391 in Barz W., Reinhard E. and Zenk M.H. M.H. 1977. Plant Tissue Culture and its Bio-technological Application. Springer-Verlag. Berlin, Heidelberg, New York.
- Zambryski P., Tempè J., Schell J. (1989). Cell 56:193:291.

24

## CLAIMS

1. Method to produce cellular vegetal bulks with an high regeneration capability comprising a chemical and a mechanical treatment, wherein the chemical treatment includes:

    a) culturing *in vitro* proliferating shoots on a first nutrition substrate comprising cytokinins till to the production of shoots to be transplanted;

    b) transplanting the shoots obtained according to a) on a second nutrition substrate comprising cytokinins at a concentration higher than the first substrate till to the production of shoots to be transplanted;

    c) if necessary, transplanting the shoots obtained according to b) on a third nutrition substrate comprising cytokinins at a concentration higher than the second substrate till to the production of shoots to be transplanted;

    d) if necessary, transplanting the shoots obtained according to c) on a fourth nutrition substrate comprising cytokinins at a concentration higher than the third substrate till to the production of shoots to be transplanted;

and wherein the mechanical treatment essentially consists of the steps of:

    e) removing from the shoots to be transplanted at each of the steps from a) to d) the apex without the removal of the base.

    f) if necessary, *in vitro* culturing thus obtained bulks.

2. Method to produce cellular vegetal bulks with an high regeneration capability according to claim 1 wherein cytokinins comprise BAP.

3. Cellular vegetal bulks with an high regeneration capability obtainable according to the method of claim 1 or 2.

4. Method to produce meristematic platforms with an high regeneration capability comprising the steps of:

    a) obtaining bulks according to claim 1 or 2;

b) sectioning the same in platforms;

c) if necessary, *in vitro* culturing thus obtained meristematic platforms.

5. Method to produce meristematic platforms with an high regeneration capability according to claim 4 wherein thus obtained platforms are about 2 mm thick.

6. Meristematic platforms with an high regeneration capability obtainable according to the method of claim 4 o 5.

7. Method to multiply vegetatively a plant including the steps of:

a) obtaining the meristematic platforms according to claim 4 or 5;

g) inducing in the meristematic platforms the regeneration of adventitious shoots by caulogenesis;

h) obtaining a plant from the adventitious shoots obtained in b).

8. Method to produce a genetically transformed plant by a transgene comprising the steps of:

a) obtaining the meristematic platforms according to claim 4 or 5;

b) transforming the cells of the meristematic platforms using the transgene;

c) maintaining the transformed meristematic platforms in selective medium for at least 3 regeneration cycles, so to obtain an increase of the number of the genetically transformed cells in the platform;

d) regenerating the plant from said platforms maintained in selective medium.

Fig. 1
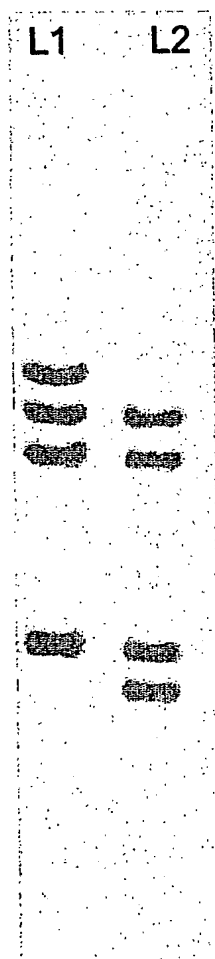
Fig. 2

(51) International Patent Classification[7]: A01H 4/00.
C12N 15/82

(21) International Application Number: PCT/IT01/00280

(22) International Filing Date: 31 May 2001 (31.05.2001)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:
RM2000A000305      5 June 2000 (05.06.2000)      IT

(71) Applicants (for all designated States except US): G.IN.E.S.TR.A. S.C. ar.l. [IT/IT]: Via Vicinale Monticino, 1869, I-47020 Diegaro di Cesena (IT). VITRO-PLANT VIVAI DI ZUCCHERELLI GIUSEPPE & C. S.S. [IT/IT]: Via Loreto, 170, I-47023 Cesena (IT).

(72) Inventors; and
(75) Inventors/Applicants (for US only): NAVACCHI, Oriano [IT/IT]: Vitroplant Vivai di Zuccherelli Giuseppe & C.S.S., Via Loreto, 170, I-47023 Cesena (IT). MEZZETTI, Bruno [IT/IT]: Università degli Studi di Ancona, Facoltà di Agraria, Via Ranieri - Monte Dago, I-60100 Ancona (IT). ZUCCHERELLI, Giuseppe [IT/IT]: Vitroplant Vivai di Zuccherelli Giuseppe & C.S.S., Via Loreto, 170, I-47023 Cesena (IT). SPENA, Angelo

|—/IT]: Facoltà di Scienze, Strada Le Grazie, I-37134 Verona (IT).

(74) Agents: CAPASSO, Olga et al.; Ing. Barzanò & Zanardo Roma S.p.A., Via Piemonte, 26, I-00187 Roma (IT).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report

(88) Date of publication of the international search report:
2 May 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD TO REGENERATE PLANTS AND USES THEREOF TO MULTIPLY AND/OR TRANSFORM PLANTS

(57) Abstract: A method to produce vegetal cellular bulks with high regeneration capability comprising both a chemical and a mechanical treatment and plant cell bulks obtained therefrom is described. The method of the invention is used advantageously to produce meristematic platforms with high regeneration capability, for the vegetative multiplication of a plant and achievement of a gene transformed plant.

| | |
|---|---|
| | Inter ional Application No<br>PCT/IT 01/00280 |

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7    A01H4/00        C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7    A01H    C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, EPO-Internal, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | DATABASE WPI<br>Section Ch, Week 199310<br>Derwent Publications Ltd., London, GB;<br>Class C06, AN 1993-079362<br>XP002184496<br>-& JP 05 023071 A (MITSUBISHI AGRIC MACH<br>CO LTD), 2 February 1993 (1993-02-02) | 1-3 |
| Y | abstract | 4-8 |
| X | WO 99 15003 A (UNIV CALIFORNIA ;US<br>AGRICULTURE (US))<br>1 April 1999 (1999-04-01) | 6 |
| Y |  example 4 | 4-8 |
| X | WO 95 35388 A (EPITOPE INC )<br>28 December 1995 (1995-12-28)<br> example 10 | 6-8 |
| | -/-- | |

[X] Further documents are listed in the continuation of box C.    [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 4 December 2001 | 21/01/2002 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Maddox, A |

Form PCT/ISA/210 (second sheet) (July 1992)

4

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
| X | GB 2 211 204 A (OJI PAPER CO)<br>28 June 1989 (1989-06-28)<br>page 12, line 20<br>--- | 6 |
| X | EP 0 132 414 A (MILOUDA LTD)<br>30 January 1985 (1985-01-30)<br>page 3, line 25 -page 4, line 7<br>page 9, line 25 -page 10, line 26;<br>examples 1,2<br>--- | 6 |
| X | DE 34 01 291 A (UNIV HIROSHIMA)<br>26 July 1984 (1984-07-26)<br>the whole document<br>--- | 6 |
| X | GB 2 195 656 A (OJI PAPER CO)<br>13 April 1988 (1988-04-13)<br>example 2<br>--- | 6 |
| A | WO 95 06741 A (BIOCEM ;KNITTEL NATHALIE<br>(AU); LENEE PHILIPPE (FR))<br>9 March 1995 (1995-03-09)<br>the whole document<br>--- | 8 |
| A | WO 96 04392 A (PIONEER HI BRED INT)<br>15 February 1996 (1996-02-15)<br>the whole document<br>--- | 8 |
| A | LINDSEY K ET AL: "TRANSFORMATION OF<br>SUGARBEET (BETA VULGARIS) BY AGROBACTERIUM<br>TUMEFACIENS"<br>JOURNAL OF EXPERIMENTAL BOTANY, OXFORD<br>UNIVERSITY PRESS, GB,<br>vol. 41, no. 226, May 1990 (1990-05),<br>pages 529-536, XP001020706<br>ISSN: 0022-0957<br>the whole document<br>--- | 8 |
| A | HAJELA RAVINDRA K ET AL: "A simple<br>transformation system using adventitious<br>shoot multiplication of juneberry."<br>HORTSCIENCE,<br>vol. 28, no. 4, 1993, pages 330-333,<br>XP002184492<br>ISSN: 0018-5345<br>the whole document<br>--- | 8 |
| A | WO 97 25434 A (CAMBRIDGE ADVANCED TECH<br>;MAUNDERS MARTIN JACK (GB); RICHARDSON<br>PET) 17 July 1997 (1997-07-17)<br>the whole document<br>--- | 8 |
| A | WO 98 56932 A (EDWARDS GLYN ALYN ;SHELL<br>INT RESEARCH (NL))<br>17 December 1998 (1998-12-17)<br>the whole document<br>--- | 8 |

-/--

4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
| A | DATABASE BIOSIS 'Online!<br>BIOSCIENCES INFORMATION SERVICE,<br>PHILADELPHIA, PA, US; 1990<br>BARIBAULT T J ET AL: "TRANSGENIC<br>GRAPEVINES REGENERATION OF SHOOTS<br>EXPRESSING BETA GLUCURONIDASE"<br>Database accession no. PREV199090122379<br>XP002184493<br>abstract<br>& JOURNAL OF EXPERIMENTAL BOTANY,<br>vol. 41, no. 229, 1990, pages 1045-1050,<br>ISSN: 0022-0957<br>--- | 8. |
| A | HOSHINO ET AL: "Production of transgenic<br>grapevine (Vitis vinifera L. cv.<br>Koshusanjaku) plants by co-cultivation of<br>embryogenic calli with Agrobacterium<br>tumefaciens and selecting secondary<br>embryos"<br>PLANT BIOTECHNOLOGY, JAPANESE SOCIETY FOR<br>PLANT CELL AND MOLECULAR BIOLOGY, TOKYO,,<br>JP,<br>vol. 15, no. 1, 1998, pages 29-33,30A,<br>XP000946719<br>ISSN: 1342-4580<br>the whole document<br>--- | 8: |
| A | DATABASE BIOSIS 'Online!<br>BIOSCIENCES INFORMATION SERVICE,<br>PHILADELPHIA, PA, US; 1990<br>YAE B W ET AL: "FACTORS AFFECTING LATERAL<br>SHOOT PROLIFERATION OF GRAPE CULTIVAR<br>RIZAMAT IN-VITRO"<br>Database accession no. PREV199191134755<br>XP002184494<br>abstract<br>& RESEARCH REPORTS OF THE RURAL<br>DEVELOPMENT ADMINISTRATION (SUWEON),<br>vol. 32, no. 3 HORTIC., 1990, pages 34-41,<br>ISSN: 1013-9397<br>--- | 8 |
| A | DATABASE BIOSIS 'Online!<br>BIOSCIENCES INFORMATION SERVICE,<br>PHILADELPHIA, PA, US; 1986<br>RAO K S: "PLANTLETS FROM SOMATIC CALLUS<br>TISSUE OF THE EAST-INDIAN ROSEWOOD<br>DALBERGIA-LATIFOLIA"<br>Database accession no. PREV198682086758<br>XP002184495<br>abstract<br>& PLANT CELL REPORTS,<br>vol. 5, no. 3, 1986, pages 199-201,<br>ISSN: 0721-7714<br>----- | 1-8 |

4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

.nformation on patent family members

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|---|
| JP 5023071 | A | 02-02-1993 | NONE | | |
| WO 9915003 | A | 01-04-1999 | AU | 9506098 A | 12-04-1999 |
| | | | EP | 1017268 A1 | 12-07-2000 |
| | | | WO | 9915003 A1 | 01-04-1999 |
| WO 9535388 | A | 28-12-1995 | US | 5750870 A | 12-05-1998 |
| | | | AU | 2705995 A | 15-01-1996 |
| | | | WO | 9535388 A1 | 28-12-1995 |
| GB 2211204 | A | 28-06-1989 | FR | 2621780 A1 | 21-04-1989 |
| | | | JP | 2138966 A | 28-05-1990 |
| EP 0132414 | A | 30-01-1985 | IL | 69333 A | 29-04-1986 |
| | | | AT | 37764 T | 15-10-1988 |
| | | | AU | 3115684 A | 31-01-1985 |
| | | | CA | 1203766 A1 | 29-04-1986 |
| | | | DE | 3474499 D1 | 17-11-1988 |
| | | | EP | 0132414 A2 | 30-01-1985 |
| | | | ES | 534846 D0 | 01-12-1986 |
| | | | ES | 8700897 A1 | 16-02-1987 |
| | | | JP | 61005727 A | 11-01-1986 |
| | | | US | 4855236 A | 08-08-1989 |
| | | | US | 5141866 A | 25-08-1992 |
| | | | ZA | 8405734 A | 24-04-1985 |
| | | | ZA | 8405735 A | 24-04-1985 |
| DE 3401291 | A | 26-07-1984 | JP | 1620538 C | 09-10-1991 |
| | | | JP | 3008171 B | 05-02-1991 |
| | | | JP | 59132823 A | 31-07-1984 |
| | | | CA | 1217339 A1 | 03-02-1987 |
| | | | DE | 3401291 A1 | 26-07-1984 |
| | | | FR | 2539579 A1 | 27-07-1984 |
| GB 2195656 | A | 13-04-1988 | JP | 1881836 C | 10-11-1994 |
| | | | JP | 6000031 B | 05-01-1994 |
| | | | JP | 63301784 A | 08-12-1988 |
| | | | JP | 1936387 C | 26-05-1995 |
| | | | JP | 6011210 B | 16-02-1994 |
| | | | JP | 63007720 A | 13-01-1988 |
| | | | JP | 2649641 B2 | 03-09-1997 |
| | | | JP | 5304849 A | 19-11-1993 |
| | | | FR | 2606788 A1 | 20-05-1988 |
| | | | FR | 2610639 A1 | 12-08-1988 |
| | | | GB | 2231585 A ,B | 21-11-1990 |
| | | | US | 5310673 A | 10-05-1994 |
| WO 9506741 | A | 09-03-1995 | FR | 2709496 A1 | 10-03-1995 |
| | | | AU | 695398 B2 | 13-08-1998 |
| | | | AU | 7539694 A | 22-03-1995 |
| | | | EP | 0716706 A1 | 19-06-1996 |
| | | | WO | 9506741 A1 | 09-03-1995 |
| | | | HU | 74664 A2 | 28-01-1997 |
| | | | JP | 9509302 T | 22-09-1997 |
| | | | SK | 25896 A3 | 04-09-1996 |
| WO 9604392 | A | 15-02-1996 | US | 5736369 A | 07-04-1998 |
| | | | AU | 697373 B2 | 01-10-1998 |

Form PCT/ISA/210 (patent family annex) (July 1992)

# INTERNATIONAL SEARCH REPORT

.iformation on patent family members

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|---|
| WO 9604392 | A | | AU | 3624795 A | 04-03-1996 |
| | | | BR | 9508341 A | 12-08-1997 |
| | | | CA | 2195206 A1 | 15-02-1996 |
| | | | EP | 0772687 A2 | 14-05-1997 |
| | | | HU | 76841 A2 | 28-11-1997 |
| | | | JP | 10503374 T | 31-03-1998 |
| | | | NZ | 293737 A | 28-07-1998 |
| | | | WO | 9604392 A2 | 15-02-1996 |
| | | | ZA | 9506302 A | 14-03-1996 |
| WO 9725434 | A | 17-07-1997 | AU | 720610 B2 | 08-06-2000 |
| | | | AU | 1388397 A | 01-08-1997 |
| | | | BR | 9706942 A | 06-04-1999 |
| | | | CN | 1213404 A | 07-04-1999 |
| | | | EP | 0880592 A2 | 02-12-1998 |
| | | | WO | 9725434 A1 | 17-07-1997 |
| | | | JP | 2000517162 T | 26-12-2000 |
| | | | ZA | 9700192 A | 23-07-1997 |
| WO 9856932 | A | 17-12-1998 | AU | 8539698 A | 30-12-1998 |
| | | | WO | 9856932 A1 | 17-12-1998 |
| | | | ZA | 9805078 A | 12-01-1999 |

BOUDREAU, Eric [CA/US]; Syngenta, 3054 Cornwallis Road, Research Triangle Park, NC 27709 (US).

(74) Agent: BASTIAN, Werner; c/o Syngenta Participations AG, Intellectual Property, P.O. Box, CH-4002 Basel (CH).

(54) Title: METHODS FOR STABLE TRANSFORMATION OF PLANTS

(57) Abstract: Multiple shoot structures are induced from plant tissues (e.g., shoot apices or axillary buds on an artificial medium) to produce multiple shoot cultures. These multi-shoot cultures are then transformed by known transformation methods. Plants are subsequently regenerated from the transformed cells. Crops that may be efficiently transformed by this method include plants normally recalcitrant to transformation such as sugar beet, sunflower, soybean, cotton, tobacco, tomato, peanuts, melons, watermelon, squash, Brassica, and pepper.

## METHODS FOR STABLE TRANSFORMATION OF PLANTS

The present invention relates to the transformation and regeneration of transformed plants. In particular, the invention relates to the transformation of plants normally recalcitrant to

5     transformation.

Improvement of plant varieties through genetic transformation has become increasingly important for modern plant breeding. Genes of potential commercial interest, such as genes conferring to a plant traits of disease resistance, insect resistance or improved quality, may

10    be incorporated into crop species through various gene transfer technologies.

The development of an efficient transformation system is necessary for the analysis of gene expression in plants. The requirements for such a system include a proper target plant tissue that will allow efficient plant regeneration, a gene delivery vehicle that delivers foreign DNA efficiently into the target plant cells, and an effective method for selecting transformed cells.

15    In genetic transformation of dicotyledonous species, for example, transformation systems utilizing the bacterium *Agrobacterium tumefaciens* have been frequently used as vehicles for gene delivery. The preferred target tissues for *Agrobacterium*-mediated transformation presently include cotyledons, leaf tissues, and hypocotyls. High velocity microprojectile bombardment offers an alternative method for gene delivery into plants.

20    Although genetic transformation and subsequent regeneration is largely a matter of routine nowadays for many plants species, several commercially significant crops such as sugar beet, squash, sunflower, soybean, and cotton have remained recalcitrant to transformation by most of the numerous methods that are available.

*Beta vulgaris* (which includes sugar beet, fodder beet, table beet and Swiss chard) is one

25    example where, despite transient expression in some cells and occasional success with specific genotypes, no simple routine method is available for the production of transgenic plants. The recalcitrance to transformation of sugar beet protoplasts is well-documented. *See*, for example, International Patent Application No. WO 91/13159 and K. D'Halluin et. al., *Bio/Technology*, 10 309-314 (1992)). Regarding *Agrobacterium*-mediated gene transfer, "the

30    sugarbeet's recalcitrance is renowned," and "most of the techniques reported for the production of transgenic sugarbeet plants require the expert skill of laboratories that developed them and [has] proved not easily reproducible by others." F. A. Krens et al., *Plant Sci.* 116, 97-106 (1996), citing M. C. Elliot et al., in *Physiology and Biochemistry of Cytokinins in Plants*, pages 329-334 (SPB Publishing, The Hague, 1992); K. Lindsey et al., *J.*

35    *Exp. Bot.* 41, 529-536 (1990); and D'Halluin et al., *supra*. The Krens et al. paper states further that "the method of choice . . . is one using cotyledons as the explant system, which

has only been described superficially." Krens et al., *supra*, citing J. E. Fry et al., Abstract # 384 in *Molecular Biology of Plant Growth and Development, Third International Congress of the International Society for Plant Molecular Biology* (R. B. Hallick, editor, Tuscon, Arizona, USA 1991). However, even this method is not highly efficient; as an example, one

5     researcher reported obtaining only 21 transgenic shoots, including several chimeras, from 15,000 inoculated explants. U. Sander, *Transformation von Beta vulgaris L.*, (Ph.D. Thesis, University of Hanover, Germany 1994). The Krens et al. paper itself reports a 0.9% transformation frequency in sugarbeets using a cotyledonary node and kanamycin selection technique. Krens et al., *supra*, at page 103.

10    There are brief descriptions in the literature relating to callus production from the epidermal cells of sugar beet (*see* Kotowska et al., *Bull. of the Polish Acad. Sci.* **32**, 11-12 (1984); Kotowska, *Beitr. Biol. Pflanz.* **67**, 209-223 (1992)), and several reports describing adventitious bud production on the epidermis of sugar beet petioles. *See e.g.*, Harms et. al., *Plant Cell Tissue Organ Culture* **2**, 93-102 (1983); Schneider et al., *Biochem. Physiol. Pflanz.*

15    **182**, 485-490 (1987)). However, these reports present no evidence that transformed sugar beet plants can be regenerated using these methods.

Sugarbeet transformation using sugarbeet protoplasts (through stomata guard cells) has been reported. *See, e.g.*, R. Hall et al., *Nature Biotechnology* **14**, 1133-1138 (1996); R. Hall et al., *Plant Physiol.* **107**, 1379-1386 (1995); R. Sevenier et al., *Nature Biotechnology* **16**,

20    843-846 (1998); and European Patent EP 0 723 393 B1. However, protoplasts isolated from sugar beet leaves vary in size and morphology, reflecting the high degree of cellular heterogeneity present within the source tissue at both physiological and cytogenetic levels. Accordingly, transformation techniques utilizing protoplasts requires the expert skill of laboratories that have developed particular methods, and the results are not easily

25    reproducible.

At best, transformation techniques for sugarbeets have heretofore been very dependent on explant source, plant genotype and selection conditions used, and high efficiencies of transformation have been very difficult to achieve. *See, e.g.*, K. Lindsey et al., *J. Experimental Botany* **41**, 529-536 (1990); K. Lindsey et.al, "Transformation in Sugar Beet

30    (*Beta vulgaris L.*)," in *Biotechnology in Agriculture and Forestry, Vol. 23, Plant Protoplasts and Genetic Engineering IV* (Y. P. S. Bajaj, Ed., Springer-Verlag, Berlin, 1993). Sugar beet is an important crop in the temperate climate region. Over 30% of the world's sugar consumption comes from sugar beet. There is thus a continuing need for a simple, high efficiency transformation method which may be applicable to beet and other plants that have

35    heretofore been recalcitrant to transformation.

Other plants recalcitrant to transformation for which simple, high efficiency transformation methods are needed include the various species of squash. In one study, summer squash cultivars were regenerated via somatic embryogenesis using cotyledons excised from seeds. C. Gonsalves, *HortScience* **30**, 1295-1297 (1995). However, the regeneration efficiency of this method was calculated as 0.3 plantlets per initial explant. *Id.* In other reports, the production of transgenic squash is reported, but the transformation procedures used to obtain the regenerated plants are not described or publicly available. *See, e.g.,* D. Tricoli et al., *Bio/Technology* **13**, 1458-1465, 1464 (1995).

Transformation methods using seedling (non-excised) shoot tips or excised shoot tips have been described. *See* U.S. Patent No. 5,164,310 to Smith et al. (incorporated herein by reference in its entirety); P. Chee et al., *Plant Physiol.* **91**, 1212-1218 (1989); F. J. L. Aragao et al., *Int. J. Plant Sci.* **158**, 157-163 (1997); and P. Christou et al., *Plant J.* **8**, 275-281 (1992). In these methods, shoot tips either remained attached to seedlings or germinating seeds at the time of transformation, or were excised from seedlings. In the latter case, only one shoot per excised shoot tip was ultimately produced.

Transformation methods using shoot tip-derived meristematic cultures have also been described. *See, e.g.,* U. S. Patent No. 5,767,368 to Zhong et al. (incorporated herein by reference in its entirety); H. Zhong et al., *Plant Physiol.* **110**, 1097-1107 (1996); and S. Zhang et al., *Plant Cell Reports* **18**, 959-966 (1999). However, these methods have been shown to be effective in only a handful of monocotyledonous plants (*i.e.*, corn, barley and oat), and no transformed dicot plants have been successfully regenerated following transformation attempts using those methods. Furthermore, transformation of monocots using shoot-tip derived meristematic cultures, as described in the above references, were generally achieved by microprojectile bombardment (*i.e.*, generally not mediated by *Agrobacterium tumefaciens*).

The present inventors have discovered a method by which multiple shoot structures are induced from plant tissues (*e.g.*, shoot apices or axillary buds on an artificial medium) to produce multiple shoot cultures. These multi-shoot cultures are then transformed with *Agrobacterium tumefaciens*, high velocity bombardment with DNA-coated microprojectiles, or other known transformation methods. After transformation, the multiple shoot cultures may be transferred to a selection medium to differentiate transformed and non-transformed cells. Plants are subsequently regenerated from the transformed cells upon transfer to a regeneration medium and/or a rooting medium. The present invention may be applied to commercial crops that include sugar beet, sunflower, soybean, cotton, peanuts, melons,

-3-

watermelon, squash, *Brassica*, tobacco, tomato and pepper. The present invention provides certain advantages over existing methods because it can be used to transform plants that are normally recalcitrant to transformation at high efficiencies. The present invention invention is also used to transform the plastid genome of plants, in particular of

5      dicotyledonous plants.

The present invention provides:

A method of producing a transformed dicotyledonous plant, comprising: (a) culturing a tissue of a dicotyledonous plant recalcitrant for transformation on a culture medium to produce a

10     multiple shoot culture from the tissue; (b) introducing a nucleic acid into a cell of the multiple shoot culture, thereby producing a transformed cell comprising the nucleic acid; and (c) regenerating a transformed plant from the transformed cell. The tissue is preferably a meristematic tissue, preferably excised from a shoot apex, an axillary bud or a floral meristem, or the tissue is callus tissue. IN a preferred embodiment, the plant is a member of

15     the *Cucurbitaceae* family or the *Chenopodiacea* family. In another preferred embodiment, the plant is selected from the group consisting of sugar beet, sunflower, soybean, cotton, melons, watermelon, and squash. Preferably, the plant is sugar beet, squash, melon or watermelon. In another preferred embodiment, the tissue is excised from the shoot tip of a seedling of the plant. In another preferred embodiment, the culture medium comprises at

20     least one plant growth regulator, preferably a cytokinin. In another preferred embodiment, the growth regulator is selected from the group consisting of 6-furfurylaminopurine (kinetin), 6-benzyl-aminopurine (6-BAP), 6-dimethyallylamino-purine (2ip), trans-6-(4-hydroxzy-3-methlbut-2-enyl) amino-urine (zeatin), TDZ, gibberellic acid (GA), IAA, NAA, dicamba, 2,3,5-T and 2,4-D. The concentration of growth regulator in the culture medium is preferably

25     between about 0.01 mg/L to about 25 mg/L, more preferably between about 0.01 mg/L to about 10 mg/L, more preferably between about 0.01 mg/L to about 5 mg/L, more preferably between about 0.05 mg/L to about 8 mg/L. In another preferred embodiment, the nucleic acid is introduced into the cell by microparticle bombardment, electrophoresis or electroporation, or using a bacterium belonging to the genus *Agrobacterium*. In another preferred

30     embodiment, the nucleic acid comprises a nucleic acid that is heterologous to the dicotyledonous plant. Preferably, the nucleic acid comprises a gene that encodes a polypeptide having PPO activity, a gene that encodes a polypeptide having phosphomannose isomerase (PMI) activity, a gene that encodes a polypeptide having xylose isomerase (xylA) activity or a gene that encodes a polypeptide having GUS activity.

-4-

Preferably, the nucleic acid is a vector comprising a nucleic acid comprising a gene heterologous to the plant.

In yet another preferred embodiment, step (c) of the method comprises: selecting a multiple shoot culture comprising a transformed cell; growing the multiple shoot culture under

5      conditions that promote shoot elongation to produce at least one transformed shoot; and then growing the at least one transformed shoot into a mature transformed plant. Preferably, the at least one transformed shoot grows into a mature transformed plant after growing the at least one transformed shoot on a medium that promotes root formation.

In yet another preferred embodiment, step (c) of the method comprises: selecting a multiple

10     shoot culture comprising a transformed cell; growing the multiple shoot culture under conditions that promote shoot elongation to produce at least one transformed shoot; cloning the at least one transformed shoot; and allowing the cloned shoot to mature into a transformed plant. Preferably, the cloned shoot grows into a mature transformed plant after growing the cloned shoot on a medium that promotes root formation.

15

The present invention further provides:

A transformed plant cell produced by any one of the methods above.

A multiple shoot culture produced by any one of the methods above.

A transformed plant produced by any one of the methods above. In a preferred embodiment,

20     the transformed plant is a squash plant, a melon plant or a watermelon plant that expresses a polypeptide having PMI activity. In another preferred embodiment, the transformed plant is a sugar beet plant that expresses a polypeptide having PPO activity.


The present invention further provides:

25     A seed produced by a transformed plant above, wherein the seed comprises the nucleic acid transformed into the multiple shoot culture and a plant grown from the seed.


The present invention further provides:

A method of producing a plant comprising a transformed plastid genome, comprising: (a)

30     culturing a tissue of a plant on a culture medium to produce a multiple shoot culture from the tissue; (b) introducing a nucleic acid into a plastid genome of a cell of the multiple shoot culture, thereby producing a transformed plastid genome of said cell comprising the nucleic acid; and (c) regenerating a transformed plant from the transformed cell. In a preferred embodiment, the tissue is meristematic tissue, preferably is excised from a shoot apex, an

35     axillary bud, a floral meristem or leaf tissue, or the tissue is callus tissue. In a preferred

embodiment, the transformed cell is homoplasmic for transformed plastid genomes. In another preferred embodiment, the plant is homoplasmic for transformed plastid genomes. In another preferred embodiment, the plant is a dicotyledonous plant. Preferably, the plant is a member of the *Cucurbitaceae* family or of the *Chenopodiacea* family. In another preferred embodiment, the plant is selected from the group consisting of sugar beet, sunflower, soybean, cotton, peanuts, melons, watermelon, tobacco, tomato, squash, *Brassica* and pepper. Preferably, the plant is sugar beet, tobacco and tomato. In another preferred embodiment, the tissue is excised or derived from the shoot tip of a seedling of the plant. In another preferred embodiment, the culture medium comprises at least one plant growth regulator, preferably a cytokinin. In another preferred embodiment, the growth regulator is selected from the group consisting of 6-furfurylaminopurine (kinetin), 6-benzyl-aminopurine (6-BAP), 6-dimethyallylamino-purine (2ip), trans-6-(4-hydroxzy-3-methlbut-2-enyl) amino-urine (zeatin), TDZ, gibberellic acid (GA), IAA, NAA, dicamba, 2,3,5-T and 2,4-D. Preferably, the concentration of growth regulator in the culture medium is between about 0.01 mg/L to about 25 mg/L, more preferably between about 0.01 mg/L to about 10 mg/L, more preferably between about 0.01 mg/L to about 5 mg/L, more preferably between about 0.05 mg/L to about 8 mg/L. The nucleic acid is preferably introduced into the cell by microparticle bombardment, electrophoresis or electroporation. Preferably, the nucleic acid comprises a nucleic acid that is heterologous to the dicotyledonous plant. Preferably, the nucleic acid is a vector comprising a nucleic acid comprising a gene heterologous to the dicotyledonous plant. In another preferred embodiment, step (c) of the method comprises: selecting a multiple shoot culture comprising a transformed cell; growing the multiple shoot culture under conditions that promote shoot elongation to produce at least one transformed shoot; and then growing the at least one transformed shoot into a mature transformed plant. In a preferred embodiment, the at least one transformed shoot grows into a mature transformed plant after growing the at least one transformed shoot on a medium that promotes root formation.

In another preferred embodiment, step (c) of the method comprises: selecting a multiple shoot culture comprising a transformed cell; growing the multiple shoot culture under conditions that promote shoot elongation to produce at least one transformed shoot; cloning the at least one transformed shoot; and allowing the cloned shoot to mature into a transformed plant. Preferably, the cloned shoot grows into a mature transformed plant after growing the cloned shoot on a medium that promotes root formation.

The present invention further provides:

A transformed plastid genome produced by any one of the methods above.

A plastid comprising a transformed plastid genome produced by any one of the methods above.

A transformed plant cell produced by any one of the methods above. Preferably, the plant cell is homoplasmic for the transformed plastid genome.

A multiple shoot culture produced by any one of the methods above.

A transformed plant produced by any one of the methods above. Preferably, the plant is homoplasmic for the transformed plastid genome.

The present invention further provides:

A seed produced by a transformed plant above, wherein the seed comprises the nucleic acid transformed into the multiple shoot culture and a plant grown from the seed.

The foregoing and other aspects of the present invention are explained in detail in the specification set forth below.

The present invention will now be described more fully hereinafter with reference to the accompanying specification, in which preferred embodiments of the invention are described. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used in the description of the invention and the appended claims, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

Except as otherwise indicated, standard methods may be used for the production of cloned genes, expression cassettes, vectors (e.g., plasmids), proteins and protein fragments, and transformed cells and plants according to the present invention. Except as otherwise indicated, standard methods may be used for the production of cloned genes, expression cassettes, vectors (e.g., plasmids), proteins and protein fragments according to the present

-7-

invention. Such techniques are known to those skilled in the art. *See e.g.*, J. Sambrook et al., *Molecular Cloning: A Laboratory Manual Second Edition* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989), and F. M. Ausubel et al., *Current Protocols In Molecular Biology* (Green Publishing Associates, Inc. and Wiley-Interscience, New York, 1991); J. Draper et al., eds., *Plant Genetic Transformation And Gene Expression: A Laboratory Manual,* (Blackwell Scientific Publications, 1988); and S.B. Gelvin & R.A. Schilperoort, eds., *Introduction, Expression, And Analysis Of Gene Production In Plants.* The methods of the present invention are useful for producing transformed plants. Transformed plants of the present invention are produced by first culturing a plant tissue on a culture medium in order to produce a multiple shoot culture from the tissue. A nucleic acid is then introduced into a cell of the multiple shoot culture, thereby producing a transformed cell comprising the nucleic acid. Finally, a transformed plant from the transformed cell is regenerated.

In a preferred embodiment of the invention, plant cells that are transformed are normally recalcitrant to transformation. In a particularly preferred embodiment, plant cells that are transformed are dicotyledonous plant cells, and most preferably are dicotyledonous plant cells recalcitrant to transformation as defined herein. "Plant cells" or "plants" as used herein includes whole plants, plant cells, or sub-cellular plant organelles (*i.e.*, plastids) in plant tissue, or plant tissue, plant cells, plant cell suspensions, sub-cellular plant organelles and protoplasts in culture. Plant tissue includes differentiated and undifferentiated tissues of plants, including but not limited to, roots, shoots, leaves, pollen, seeds, tumor tissue, gametophytes, sporophytes, microspores and various forms of cells in culture, such as single cells, protoplasts, embryos and callus tissue. The plant tissue may be in a plant, or in an organ, tissue or cell culture.

In general, dicotyledonous plants are flowering plants having an embryo with two cotelydons, leaves with net-like veins, and vascular bundles in the stem in a ring surrounding the central pith. As stated above, dicotyledonous plants that may be transformed by the present invention are preferably those dicotyledonous plants that are recalcitrant to transformation. Generally, a plant recalcitrant to transformation is one whose cells either cannot be transformed by present known transformation methods (*i.e.*, microprojectile bombardment or biolistic transformation, *Agrobacterium*-mediated transformation, electroporation, electrophoresis and the like), or is transformed by such methods at a low transformation efficiency (*i.e.*, lower than 1.0%, or lower than 0.5%, or even lower than 0.1%). Plants recalcitrant to transformation are also those plants whose cells, tissue, protoplasts or calli

may be transformed by known methods, but which transformed cells, tissue, protoplasts or calli are unable to regenerate a mature transgenic plant.

In a preferred embodiment of the invention, the dicotyledonous plant is a member of the *Cucurbitaceae* family. In another preferred embodiment of the invention, the dicotyledonous

5     plant is a member of the *Chenopodiacea* family. In a more preferred embodiment, the dicotyledonous plant is selected from the group consisting of *Beta vulgaris* (*i.e.*, sugar beet, fodder beet, table beet and Swiss chard), sunflower, soybean, cotton, peanuts, melons, watermelon, squash, tobacco, tomato, *Brassica* and pepper. In a most preferred embodiment, the dicotyledonous plant is sugar beet or squash.

10    In the present invention, a plant is transformed by first producing a multiple shoot culture from tissue from the plant. As used herein, the term "multiple shoot culture" may be also referred to interchangeably as an "explant." Tissue that may be utilized in the present invention includes but is not limited to meristematic tissue, root tip tissue, shoot tip tissue (including leaf primordia, apical regions, axillary regions, hypocotyl and cotyledonous leaves),

15    seedling shoot apex (which may include meristem, leaf blade, leaf petiole, or blade-petiole transition zone tissue), and callus tissue. In a preferred embodiment, the tissue from which a multiple shoot culture is produced from meristematic tissue excised from a shoot apex, an axillary bud or a floral meristem.

In one embodiment of the invention, a multiple shoot culture may be obtained by first

20    germinating seeds on a seed germination medium, preferably under aseptic culture. Seed germination techniques are known to those skilled in the art. In a preferred embodiment of the invention, the seed germination medium (GM) comprises Murashige and Skoog (MS) salts (commercially available from Sigma Chemicals, St. Louis, Mo.). The GM may further comprise a carbohydrate source such as sucrose, which is preferred. The carbohydrate

25    source may be present in an amount of about 20-40 g/L (about two to four percent w/v), and preferably about 30 g/L (about three percent w/v). Optionally and preferably, the GM may comprise $B_5$ vitamins, myo-inositol (100mg), pantothenic acid and a plant tissue culture gelling agent such PHYTAGEL™ (also available from Sigma Chemical). Plant growth regulators with cytokinin-like function (described below) may also (and preferably) be added

30    to the seed germination medium. Suitable plant growth regulators with cytokinin-like function include but are not limited to 6-benzyl-aminopurine (6-BAP or BA), 6-furfurylaminopurine (kinetin), 6-imethyallylamino-purine (2-ip), trans-6-(4-hydroxzy-3-methylbut-2-enyl) amino-urine (zeatin) and the like. In GM, the plant growth regulator may be added to a concentration as low as about 0.5mg/L or about 0.1 mg/L, or about 0.05 mg/L, or even lower;

35    alternatively, the plant growth regulator may be added to a concentration of up to about 1.0

mg/L or about 2.0 mg/L, or about 5 mg/L, or even higher.  The addition of casein hydrolysate, proline and/or glutamin may also be beneficial.  For some dicotyledonous crops such as sugar beet, the addition to the GM of an auxin inhibitor such as 2,3,5-triiodobenzoic acid (TIBA) may be beneficial.

After the seeds have germinated (*i.e.,* become seedlings) on the GM for about 7-14 days, the shoot tips of the seedlings are excised and cultured (i.e., plated) on a culture medium referred to herein as a shoot multiplication medium (SMM).  Preferably, the shoot tip comprises both apical and axillary meristem regions, leaf primordia, 3-4 mm of hypocotyl, and the cotyledons, which may be cut back to reduce further elongation.  Of course, other plant tissue used in the present invention to produce multiple shoot cultures may also be plated on the SMM.  In a preferred embodiment of the invention, the SMM comprises MS salts, a carbohydrate source (preferably sucrose), $B_5$ vitamins and a gelling agent such as PHYTAGEL™.  In addition, the SMM contains at least one cytokinin-like growth regulator such as BA, kinetin, 2ip, zeatin and the like.  BA is the preferred growth regulator for inducing shoot meristematic cultures from apical or axillary meristems of sugar beet or other dicotyledonous species on sucrose containing medium.  The cytokinin-like growth regulator may be present in the SMM at a concentration as low as about 2.0 mg/L, or about 1.0 mg/L, or about 0.05mg/L or about 0.01 mg/L, or even lower; alternatively, the cytokinin-like growth regulators may be present in the SMM at a concentration as high as about 5 mg/L, or about 8 mg/L, or 10 mg/L, or about 25, or about 100mg/L, or even higher.  In one embodiment, the cytokinin-like growth regulator is present in the SMM in from about 0.05 mg/L to about 25 mg/L, more preferably from 0.1 mg/L to 10 mg/L, and most preferably from about 0.5 mg/L to about 8 mg/L. Additional growth regulators may be added to the SMM to induce shoot meristematic cultures.  Such growth regulators include (gibberellic acid (GA), and those with auxin-like function such as indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), thidiazuron (TDZ), 3, 6-dichloro-o-anisic acid (DICAMBA), 2,4,5,-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenoxyacetic acid(2,4-D), and other growth regulators known to those skilled in the art.

The multiple shoot cultures that grow on the SMM are cultured under light or dark conditions at a temperature between 15-35°C, preferably cultured under low light intensity (from about 10- to about 30μEinsteins) for about 12 to 16 hour day-lengths at a temperature of about 20°C.  After about 4 to 6 weeks. The multiple shoot cultures resemble compact rosettes and are ready for transformation.

Multiple shoot cultures may be transformed with any one of many known transformation methods. As used herein, the term "transformation" refers to the stable introduction of a

-10-

nucleic acid segment generally, a heterologous nucleic acid sequence or gene) into a plant, plant tissue, plant organelle (i.e., a plastid or chloroplast), or plant cell that did not previously contain that gene. Preferably, transformation results in the stable integration of the nucleic acid sequence into the genome of the plant. As used herein, the term "genome"

5    encompasses nuclear genomes, plastid genomes, and mitochondrial genomes.

The inventors of the present invention have also discovered that large amounts of plastids are present in the cells of multiple shoot cultures. Such cultures thus provide an interesting target tissue for plastid transformation. Consequently, in an alternative preferred embodiment, a method of the present invention is used to transform the plastid genome of a

10   plant cell. Preferably, the plastid genome of a dicotyledonous plant cell is transformed using a method of the present invention. In another preferred embodiment, the plastid genome of a dicotyledonous plant cell normally recalcitrant to transformation is transformed using a method of the present invention.

A major advantage of plastid transformation is that plastids are generally capable of

15   expressing bacterial genes without substantial modification, and plastids are capable of expressing multiple open reading frames under control of a single promoter. Plastid transformation technology is described in U.S. Patent Nos. 5,451,513; 5,545,817; and 5,545,818; in PCT Application No. WO 95/16783; and in McBride et al., *Proc. Natl. Acad. Sci. USA* **91**, 7301-7305 (1994). The basic technique for chloroplast transformation involves

20   introducing regions of cloned plastid DNA flanking a selectable marker together with the gene of interest into a suitable target tissue, *e.g.*, using biolistics or other protoplast transformation method such as calcium chloride or PEG-mediated transformation. The 1 to 1.5 kb flanking regions, termed targeting sequences, facilitate homologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of

25   the plastome. Initially, point mutations in the chloroplast 16S rRNA and rps12 genes conferring resistance to spectinomycin and/or streptomycin are utilized as selectable markers for transformation. *See* Z. Svab et al., *Proc. Natl. Acad. Sci. USA* **87**, 8526-8530 (1990), and J. M. Staub et al., *Plant Cell* **4**, 39-45 (1992). The presence of cloning sites between these markers allowed creation of a plastid targeting vector for introduction of foreign genes. *See*

30   J. M. Staub, et al., *EMBO J.* **12**, 601-606 (1993). Substantial increases in transformation frequency are obtained by replacement of the recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial aadA gene encoding the spectinomycin-detoxifying enzyme aminoglycoside-3'-adenyltransferase. *See* Z. Svab et al., *Proc. Natl. Acad. Sci. USA* **90**, 913-917 (1993). Previously, this marker had been used

35   successfully for high-frequency transformation of the plastid genome of the green alga

-11-

*Chlamydomonas reinhardtii.* M. Goldschmidt-Clermont, *Nucl. Acids Res.* 19, 4083-4089 (1991). Other selectable markers useful for plastid transformation are known in the art. Plastid expression, in which genes are inserted by homologous recombination into the several thousand copies of the circular plastid genome present in each plant cell, takes

5    advantage of the enormous copy number advantage over nuclear-expressed genes to permit expression levels that can readily exceed 10% of the total soluble plant protein. In a preferred embodiment of the invention, a nucleotide sequence of interest is inserted into a plastid targeting vector and transformed into the plastid genome of a desired plant host. Plants homoplasmic for plastid genomes containing the nucleotide sequence of interest are

10   preferably obtained, and are preferentially capable of high expression of the nucleotide sequence.

The term "heterologous" is used herein to indicate a nucleic acid sequence (*e.g.*, a gene) or a protein has a different natural origin (*i.e.*, from a different species, or from the same species but in a different chromosomal location, orientation, or copy number) with respect to its

15   current host. "Heterologous" is also used to indicate that one or more of the domains present in a protein differ in their natural origin with respect to other domains present. The term "nucleic acid" as used herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, nucleic acid analogs are included that may have alternate backbones. In

20   addition, mixtures of naturally occurring nucleic acids and analogs and non-naturally occurring nucleic acids and analogs may be used. Nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA, or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any

25   combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, *etc.*

The transformation method used for a given plant species or specific type of plant tissue depends on the most appropriate method as determined by the skilled artisan, based on known methods. As novel means are developed for the stable insertion of foreign genes into

30   plant cells and for manipulating the modified cells, skilled artisans will be able to select from known means to achieve a desired result. Known transformation techniques useful in the practice of the present invention include, but are not limited to, microparticle bombardment (U.S. Patent No. 4,945,050 to Sanford *et al.* and McCabe *et al., Bio/Technology* 6, 923-926); direct DNA uptake (J. Paszkowski et al., *EMBO J.* 3,2717 (1984)); electroporation (M. Fromm

35   *et al. Proc. Natl. Acad. Sci. USA* 82,5824 (1985); microinjection (A. Crossway *et al. Mol.*

-12-

*Gen. Genet.* **202**, 179 (1986)); and *Agrobacterium*-mediated gene transfer to the plant tissue. In the present invention, microparticle bombardment and *Agrobacterium*-mediated transformation techniques are preferred, with *Agrobacterium*-mediated gene transfer being most preferred.

*Agrobacterium*-mediated gene transfer exploits the natural ability of the bacteria *A. tumefaciens* and *A. rhizogenes* to transfer DNA into plant chromosomes. *Agrobacterium* is a plant pathogen that transfers a set of genes encoded in a region called T-DNA of the Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, into plant cells. Transformation using *A. rhizogenes* developed analogously to that of *A. tumefaciens*. *See* U.S. Patent No. 5,777,200 to Ryals et al., the disclosure of which is incorporated herein by reference in its entirety. The typical result of transfer of the Ti plasmid is a tumorous growth called a crown gall in which the T-DNA is stably integrated into a host chromosome. Integration of the Ri plasmid into the host chromosomal DNA results in a condition known as "hairy root disease." The ability to cause disease in the host plant can be removed by deletion of the genes in the T-DNA without loss of DNA transfer and integration. The nucleic acid to be delivered to a plant cell for purposes of transformation (*i.e.*, a heterologous gene) is attached to border sequences that define the end points of an integrated T-DNA.

Any suitable *Agrobacterium* vector or vector system for transforming the plant may be employed according to the present invention. A variety of *Agrobacterium* strains are known in the art and may be used in the methods of the invention. Representative *Agrobacterium* vector systems are described in G. An, et al. *EMBO J.* **4**, 277 (1985); L. Herrera-Estrella, et al., *Nature* **303**, 209 (1983); L. Herrera-Estrella *et al.*, *EMBO J.* **2**, 987 (1983); L. Herrera-Estrella et al., in *Plant Genetic Engineering* (Cambridge University Press, New York, page 63 (1985); Hooykaas, *Plant Mol. Biol.* **13**, 327 (1989); Smith et al., *Crop Science* **35**, 301 (1995); Chilton, *Proc. Natl. Acad. Sci. USA* **90**, 3119 (1993); Mollony et al., *Monograph Theor. Appl. Genet. NY* **19**, 148 (1993); Ishida et al., *Nature Biotechnol.* **14**, 745 (1996); and Komari et al., *The Plant Journal* **10**, 165 (1996), the disclosures of which are incorporated herein by reference in their entirety.

In addition to the T-region of Agrobacterium, the Ti (or Ri) plasmid contains a vir region. The vir region is important for efficient transformation, and appears to be species-specific. Binary vector systems have been developed where the manipulated disarmed T-DNA carrying, for example, heterologous DNA and the vir functions are present on separate plasmids. In other words, a heterologous nucleic acid sequence (*i.e.*, gene or genes) of interest and the flanking T-DNA can be carried by a binary vector lacking the vir region. The vir region is then provided on a disarmed Ti- plasmid or on a second binary plasmid. In this manner, a

-13-

modified T-DNA region comprising heterologous DNA is constructed in a small plasmid which replicates in E. coli. This plasmid is transferred conjugatively in a tri-parental mating or via electroporation into A. tumefaciens that contains a compatible plasmid with virulence gene sequences. The vir functions are supplied in trans to transfer the T-DNA into the plant genome. As another alternative, the heterologous nucleic acid sequence and the T-DNA border sequences can be put into the T-DNA site on the Ti-plasmid through a double recombination event by which the new T-DNA replaces the original Ti-plasmid T-DNA. The vir region can be supplied by the Ti-plasmid or on a binary plasmid. As yet a further alternative, the heterologous nucleic acid sequence and flanking T-DNA can be integrated into the bacterial chromosome as described by U.S. Patent No. 4,940,838 to Schilperoort et al., and the vir region can then be supplied on a Ti-plasmid or on a binary plasmid. Binary vectors as described herein may be used in the practice of the present invention, and are preferred.

Alternatively, in other embodiments of the invention, super-binary or "supervirulent" Agrobacterium vectors are employed in the Agrobacterium solutions. See, e.g., U.S. Patent No. 5,591,615 and EP 0 604 662, herein incorporated by reference. Such a super-binary vector has been constructed containing a DNA region originating from the hypervirulence region of the Ti plasmid pTiBo542 (Jin et al., J. Bacteriol. 169, 4417 (1987)) contained in a super-virulent A. tumefaciens A281 exhibiting extremely high transformation efficiency (Hood et al., Biotechnol. 2, 702 (1984); Hood et al., J. Bacteriol. 168, 1283 (1986); Komari et al., J. Bacteriol. 166, 88 (1986); Jin et al., J. Bacteriol. 169, 4417 (1987); Komari, Plant Science 60, 223 (1987); ATCC Accession No. 37394.

Exemplary super-binary vectors known to those skilled in the art include pTOK162 (see Japanese Patent Appl. (Kokai) No. 4-222527, European Patent Applications EP 504,869 and EP 604,662, and United States Patent No. 5,591,616, herein incorporated by reference) and pTOK233 (see Komari, Plant Cell Reports 9,303 (1990), and Ishida et al., Nature Biotechnology 14, 745 (1996); herein incorporated by reference). Other super-binary vectors may be constructed by the methods set forth in the above references. Super-binary vector pTOK162 is capable of replication in both E. coli and in A. tumefaciens. Additionally, the vector contains the virB, virC and virG genes from the virulence region of pTiBo542. The plasmid also contains an antibiotic resistance gene, a selectable marker gene, and, if desired, a nucleic acid of interest to be transformed into the plant. Super-binary vectors of the invention can be constructed having the features described above for pTOK162. The T-region of the super-binary vectors and other vectors for use in the invention may be constructed to have restriction sites for the insertion of, for example, heterologous genes to

-14-

be delivered to the plant. Alternatively, heterologous nucleic acids to be transformed can be inserted in the T-DNA region of the vector by utilizing in vivo homologous recombination. See, Herrera-Esterella et al., *EMBO J.* 2, 987 (1983); Horch et al., *Science* 223, 496 (1984). Such homologous recombination relies on the fact that the super-binary vector has a region

5    homologous with a region of pBR322 or other similar plasmids. Thus, when the two plasmids are brought together, a desired gene is inserted into the super-binary vector by genetic recombination via the homologous regions.

Preferably, *Agrobacterium* vectors and vector systems utilized in the methods of the present invention are modified by recombinant nucleic acid techniques to contain a heterologous

10   nucleic acid (*e.g.*, a gene or genes of interest) to be expressed in the transformed cells. "Expression" refers to the transcription and translation of a structural heterologous nucleic acid to yield the encoded protein. Expression may also refer to transcription only, as for example in the case of antisense constructs. The heterologous nucleic acid to be expressed is preferably incorporated into the T-region and is flanked by T-DNA border sequences of the

15   *Agrobacterium* vector.

In addition to coding sequences, the vectors of the present invention may also optionally comprise regulatory sequences useful or necessary for the transcription and translation of the heterologous nucleic acid. Examples of such regulatory sequences include but are not limited to transcriptional initiation regions (including promoters), enhancers, signal

20   sequences, polyadenylation sequences, introns, 5´ and 3´ noncoding regions (*i.e.*, 5´ leader sequences), terminator sequences, and other regulatory elements known to those skilled in the art. General molecular biology techniques that may be used to create the modified vectors used in the invention are well-known by those of skill in the art.

The nucleic acid to be expressed after transformation is preferably placed under the control

25   of a suitable promoter sequence. The term "promoter" refers to the nucleotide sequences at the 5´ end of a structural gene or coding sequence that directs the initiation of transcription. As used herein, the term "operatively linked" means that a promoter is connected to a DNA coding region in such a way that the transcription of that DNA coding region is controlled and regulated by that promoter. Means for operatively linking a promoter to a coding region are

30   well known in the art.

For expression in plants, suitable promoters must be chosen for the host cell, the selection of which promoters is well within the skill of one knowledgeable in the art. Promoters useful in the practice of the present invention include, but are not limited to, constitutive, inducible, temporally-regulated, developmentally regulated, chemically regulated, tissue-preferred and

35   tissue-specific promoters.

Numerous promoters are known or are found to facilitate transcription of RNA in plant cells and can be used in the DNA construct of the present invention. Examples of suitable promoters include the nopaline synthase (NOS) and octopine synthase (OCS) promoters, the light-inducible promoter from the small subunit of ribulose bis-phosphate carboxylase

5    promoters, the CaMV 35S and 19S promoters, the full-length transcript promoter from Figwort mosaic virus, histone promoters, tubulin promoters, or the mannopine synthase promoter (MAS). The promoter may also be one that causes preferential expression in a particular tissue, such as leaves, stems, roots, or meristematic tissue, or the promoter may be inducible, such as by light, heat stress, water stress or chemical application or production

10   by the plant. Exemplary green tissue-specific promoters include the maize phosphoenol pyruvate carboxylase (PEPC) promoter, small submit ribulose bis-carboxylase promoters (ssRUBISCO) and the chlorophyll a/b binding protein promoters.

Additional promoters useful in the present invention include but are not limited to one of several of the actin genes (e.g., rice actin), which are known to be expressed in most cell

15   types. Yet another constitutive promoter useful in the practice of the present invention is derived from ubiquitin, which is another gene product known to accumulate in many cell types. The ubiquitin promoter has been cloned from several species for use in transgenic plants (e.g., sunflower (Binet et al., Plant Science 79: 87-94 (1991)); and maize (Christensen et al., Plant Molec. Biol. 12, 619-632 (1989)). Other useful promoters are the U2 and U5

20   snRNA promoters from maize (Brown et al., Nucleic Acids Res. 17, 8991 (1989)) and the promoter from alcohol dehydrogenase (Dennis et al., Nucleic Acids Res. 12, 3983 (1984)). Tissue-specific or tissue-preferential promoters useful in the present invention in plants are those which direct expression in root, pith, leaf or pollen. Such promoters are disclosed in U.S. Pat. No. 5,625,136 (herein incorporated by reference in its entirety). Also useful are

25   promoters which confer seed-specific expression, such as those disclosed by Schernthaner et al., EMBO J. 7: 1249 (1988); anther-specific promoters ant32 and ant43D; anther (tapetal) specific promoter B6 (Huffman et al., J. Cell. Biochem. 17B, Abstract #D209 (1993)); and pistil-specific promoters such as a modified S13 promoter (Dzelkalns et al., Plant Cell 5,855 (1993)).

30   Other plant promoters may be obtained, preferably from plants or plant viruses, and may be utilized so long as the selected promoter is capable of causing sufficient expression in a plant resulting in the production of an effective amount of the desired protein. In a preferred embodiment of the invention, the promoter is the rice actin-1 promoter.

Any heterologous gene or nucleic acid that is desired to be expressed in a plant is suitable

35   for transformation in the present invention, and can be used in the methods of the invention.

-16-

Heterologous genes to be transformed and expressed in the plants of the present invention include but are not limited to genes that encode resistance to diseases, genes that encode resistance to insects, genes conferring nutritional value, genes conferring antifungal, antibacterial or antiviral activity, genes conferring resistance to herbicides, genes conferring

5      improved plant or nutritional traits, and the like. Alternatively, therapeutic (*e.g.*, for veterinary or medical uses) or immunogenic (*e.g.*, for vaccination) peptides and proteins can be expressed in plant cells transformed by the methods of the present invention.

The expression of structural genes is preferred. As used herein, a "structural gene" is that portion of a gene comprising a DNA segment encoding a protein, polypeptide or a portion

10     thereof, and excluding the 5′ sequence which drives the initiation of transcription. The structural gene may be one which is normally found in the cell or one that is not normally found in the cellular location wherein it is introduced (*i.e.*, is a heterologous gene). A heterologous gene may be derived in whole or in part from any source known to the art, including a bacterial genome or episome, eukaryotic, nuclear or plasmid DNA, cDNA, viral

15     DNA or chemically synthesized DNA. A structural gene may contain one or more modifications in either the coding or the untranslated regions which could affect the biological activity or the chemical structure of the expression product, the rate of expression or the manner of expression control. Such modifications include, but are not limited to, mutations, insertions, deletions and substitutions of one or more nucleotides. The structural gene may

20     constitute an uninterrupted coding sequence, or may include one or more introns, bounded by the appropriate splice junctions. The structural gene may be a composite of segments derived from a plurality of sources, either naturally occurring or synthetic, or both. When synthesizing a gene for improved expression in a host cell it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage

25     of the host cell.

Likewise, the methods of the present invention may be used to transfer any nucleic acid for controlling gene expression a plant. For example, the nucleic acid to be transferred can encode an antisense oligonucleotide. Alternately, plants of the present invention can be transformed with one or more genes to reproduce enzymatic pathways for chemical

30     synthesis or other industrial processes. Heterologous nucleic acids useful in the present invention may be naturally occurring and may be obtained from prokaryotes or eukaryotes (*e.g.*, bacteria, fungi, yeast, viruses, plants, insects, and mammals), or the nucleic acids may be synthesized in whole or in part.

In a preferred embodiment of the invention, the heterologous nucleic acid or gene delivered

35     or transformed into the dicotyledonous multiple shoot culture encodes a polypeptide having

-17-

protoporphyrinogen oxidase (PPO) activity. *See, e.g.,* U.S. Patent Nos. 5,767,373; 5,939,602; 6,023,012; and 6,084,155, the disclosures of which are incorporated herein by reference in their entirety. In another preferred embodiment, the nucleic acid or gene delivered or transformed into the dicotyledonous multiple shoot culture encodes a

5    polypeptide having phosphomannose isomerase (PMI) activity. *See e.g.,* U.S. Patent Nos. 5,767,378 and 5,994,629, incorporated herein by reference in their entirety). In yet another preferred embodiment, the nucleic acid or gene delivered or transformed into the dicotyledonous multiple shoot culture encodes a polypeptide having xylose isomerase (xylA) activity. In still another preferred embodiment, the nucleic acid or gene delivered or

10   transformed into the dicotyledonous multiple shoot culture encodes a polypeptide having GUS activity.

After effecting delivery of heterologous nucleic acid to recipient cells, plastids and/or plants by any of the methods listed above, identifying the cells and plants exhibiting successful or enhanced expression of a heterologous gene for further culturing and plant regeneration

15   generally occurs by one or more screening methods.

"Screening" generally refers to identifying the cells and/or plants exhibiting expression of a heterologous gene that has been transformed into the plant. Usually, screening is carried out to select successfully transformed seeds (*i.e.,* transgenic seeds) for further cultivation and plant generation (*i.e.,* for the production of transgenic plants). In order to improve the

20   ability to identify transformants, a selectable or screenable marker gene is normally transformed into plant along with the heterologous gene of interest. In such a case, one would then generally assay the potentially transformed cells, seeds or plants by exposing the cells, seeds, plants, or seedlings to a selective agent or agents. For example, transgenic cells, seeds or plants may be screened under selective conditions, such as by growing the

25   seeds or seedlings on media containing selective agents such as mannose or antibiotics (*e.g.,* hygromycin, kanamycin, or paromomycin), the successfully transformed plants having been transformed with genes encoding resistance to such selective agents. Alternatively, other methods (*e.g.,* exposing plants transformed with herbicide-resistant genes to herbicides such as a PPO inhibitor see above or BASTA®) are used to screen the cells, seeds, plants or

30   tissues of the plants for the desired marker gene. Alternatively, a positive selection method with for example a nucleic acid that encodes a polypeptide having phosphomannose isomerase (PMI) activity is used. In a preferred embodiment of the invention, the transformed cells are selected by growing the transformed multiple cultures on plant growth media comprising antibiotics and/or mannose.

In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible nucleic acid sequence of interest. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can 'select' for by chemical means, *i.e.*, through the use of a selective agent (*e.g.*, a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, i.e., by 'screening' (e.g., the R-locus trait). Many examples of suitable marker genes are known to the art and can be employed in the practice of the invention. The selectable marker gene may be the only heterologous gene expressed by a transformed cell, or may be expressed in addition to another heterologous gene transformed into and expressed in the transformed cell. Selectable marker genes are utilized for the identification and selection of transformed cells or tissues. Selectable marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (*NEO*) and hygromycin phosphotransferase (*HPT*), as well as genes conferring resistance to herbicidal compounds. Herbicide resistance genes generally code for a modified target protein insensitive to the herbicide or for an enzyme that degrades or detoxifies the herbicide in the plant before it can act. *See*, DeBlock et al., *EMBO J.* **6**, 2513 (1987); DeBlock et al., *Plant Physiol.* **91**, 691 (1989); Fromm et al., *BioTechnology* **8**, 833 (1990); Gordon-Kamm et al., *Plant Cell* **2**, 603 (1990). For example, resistance to glyphosphate or sulfonylurea herbicides has been obtained using genes coding for the mutant target enzymes, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetolactate synthase (ALS). Resistance to glufosinate ammonium, boromoxynil, and 2,4-dichlorophenoxyacetate (2,4-D) have been obtained by using bacterial genes encoding phosphinothricin acetyltransferase, a nitrilase, or a 2,4-dichlorophenoxyacetate monooxygenase, which detoxify the respective herbicides. Selectable marker genes include, but are not limited to, genes encoding: neomycin phosphotransferase II (Fraley et al., *CRC Critical Reviews in Plant Science* **4**, 1 (1986)); cyanamide hydratase (Maier-Greiner et al., *Proc. Natl. Acad. Sci. USA* **88**, 4250 (1991)); aspartate kinase; dihydrodipicolinate synthase (Perl et al., *BioTechnology* **11**, 715 (1993)); *bar* gene (Toki et al., *Plant Physiol.* **100**, 1503 (1992); Meagher et al., *Crop Sci.* **36**, 1367 (1996)); tryptophane decarboxylase (Goddijn et al., *Plant Mol. Biol.* **22**, 907 (1993)); neomycin phosphotransferase (*NEO*; Southern et al., *J. Mol. Appl. Gen.* **1**, 327 (1982)); hygromycin phosphotransferase (*HPT* or *HYG*; Shimizu et al., *Mol. Cell. Biol.* **6**, 1074 (1986)); dihydrofolate reductase (DHFR); phosphinothricin acetyltransferase (DeBlock et al.,

*EMBO J.* **6**, 2513 (1987)); 2,2- dichloropropionic acid dehalogenase (Buchanan-Wollatron et al., *J. Cell. Biochem.* **13D**, 330 (1989)); acetohydroxyacid synthase (United States Patent No. 4,761,373 to Anderson et al.; Haughn et al., *Mol. Gen. Genet.* **221**, 266 (1988)); 5-enolpyruvyl-shikimate-phosphate synthase (*aroA*; Comai et al., *Nature* **317**, 741 (1985)); haloarylnitrilase (WO 87/04181 to Stalker et al.); acetyl-coenzyme A carboxylase (Parker et al., *Plant Physiol.* **92**, 1220 (1990)); dihydropteroate synthase (*sul*; Guerineau et al., *Plant Mol. Biol.* **15**, 127 (1990)); and 32 kDa photosystem II polypeptide (*psbA*; Hirschberg et al., *Science* **222**, 1346 (1983)).

Also included are genes encoding resistance to chloramphenicol (Herrera-Estrella et al., *EMBO J.* **2**, 987 (1983)); methotrexate (Herrera-Estrella et al., *Nature* **303**, 209 (1983); Meijer et al., *Plant Mol. Biol.* **16**, 807 (1991)); hygromycin (Waldron et al., *Plant Mol. Biol.* **5**, 103 (1985); Zhijian et al., *Plant Science* **108**, 219 (1995); Meijer et al., *Plant Mol. Bio.* **16**, 807 (1991)); streptomycin (Jones et al., *Mol. Gen. Genet.* **210**, 86 (1987)); spectinomycin (Bretagne- Sagnard et al., *Transgenic Res.* **5**, 131 (1996)); bleomycin (Hille et al., *Plant Mol. Biol.* **7**, 171 (1986)); sulfonamide (Guerineau et al., *Plant Mol. Bio.* **15**, 127 (1990); bromoxynil (Stalker et al., *Science* **242**, 419 (1988)); 2,4-D (Streber et al., *Bio/Technology* **7**, 811 (1989)); phosphinothricin (DeBlock et al., *EMBO J.* **6**, 2513 (1987)); spectinomycin (Bretagne-Sagnard and Chupeau, *Transgenic Research* **5**, 131 (1996)).

The foregoing list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

To additionally confirm the presence of the heterologous nucleic acid or "transgene(s)" in the seeds of the transformed plant or in the regenerated plants produced from those seeds, a variety of assays may be performed. Such assays include, for example, molecular biological assays, such as Southern and Northern blotting and PCR; biochemical assays, such as detecting the presence of a protein product, by immunological means (ELISAs and Western blots) or by enzymatic function; and by plant part assays, such as leaf or root assays. While Southern blotting and PCR may be used to detect the gene(s) in question, they do not provide information as to whether the gene is being expressed. Expression of the heterologous gene may be evaluated by specifically identifying the protein products of the introduced genes or evaluating the phenotypic changes brought about by their expression. Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The

unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as Western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic

5    techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these techniques are among the most commonly employed, other procedures are known in the art and may be additionally used.

One particular advantage of the present invention is that the use of a multiple shoot culture

10   or explant for transformation, rather than a single-shoot culture, increases the efficiency of transformation of plants that are otherwise recalcitrant to transformation. By efficiency is meant the percentage of plants or plant cells successfully transformed with a desired heterologous nucleic acid, as compared to the percentage of plants or plant cells not successfully transformed with the heterologous nucleic acid. In a preferred embodiment of

15   the invention, the transformation efficiency of the method of the invention is higher than about 1%, more preferably higher than 4%, even more preferably higher than 10 %, and most preferably higher than about 30 %. The transformation efficiency is defined as the ratio between the number of explants giving a transgenic plant and the number of target explants. Transgenic plants comprising the heterologous nucleic acid of the present invention (e.g.,

20   comprising a heterologous nucleic acid of the present invention, or a transformed cell of the present invention), as well as the seeds and progeny produced by the transgenic plants are also an aspect of the present invention. Procedures for cultivating transformed cells to useful cultivars are known to those skilled in the art. Techniques are known for the in vitro culture of plant tissue, and in a number of cases, for regeneration into whole plants. A further aspect of

25   the invention is transgenic plant tissue, plants or seeds containing the nucleic acids described above. In a preferred embodiment, transformed explants produced using the present invention are not chimeric, or only a small proportion of transformed explants is chimeric. This is preferably achieved by extending the period of high cytokinin treatment or by increasing the stringency of mannose selection, or both. (See Example 5, below).

30   Thus, the transformed cells of the present invention, identified by selection or screening and cultured in an appropriate medium that supports regeneration as provided herein, may then be allowed to mature into plants. Plants are preferably matured either in a growth chamber or greenhouse. Plants are regenerated from about six weeks to ten months after a transformant is identified, depending on the initial tissue. During regeneration, cells may be

35   grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels are

-21-

petri dishes and Plant Con®s. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing. As provided above, seeds and progeny plants of the regenerated plants are an aspect of the present invention. Accordingly, the term "seeds" is meant to encompass seeds

5  of the transformed plant, as well as seeds produced from the progeny of the transformed plants. Plants of the present invention include not only the transformed and regenerated plants, but also progeny of transformed and regenerated plants produced by the present invention.

In one embodiment, regenerated transgenic plants of the present invention are made by first

10  growing transformed multiple shoot cultures on selection media and removing all non-transformed tissue as the transformed culture matures. After selection is completed, the shoots produced by the transformed cultures are transferred to containers that preferably contain a shoot elongation medium; such media are known in the art and described below. If desired, transformed shoots may be cloned on MS-based cloning medium (for example, a

15  growth medium as described above, further comprising mannose). Alternatively, a single shoot or clone may be successfully rooted by transferring the shoot or clone to an appropriate rooting medium as described below or as may be determined by the skilled artisan.

Plants produced by the methods of the present invention may be screened for successful

20  transformation by standard methods described above. Seeds and progeny plants of regenerated plants of the present invention may be continuously screened and selected for the continued presence of the transgenic and integrated nucleic acid sequence in order to develop improved plant and seed lines, which are an another aspect of the present invention. Desirable transgenic nucleic acid sequences may thus be moved (*i.e.*, introgressed or

25  inbred) into other genetic lines such as certain elite or commercially valuable lines or varieties. Methods of introgressing desirable nucleic acid sequences into genetic plant lines may be carried out by a variety of techniques known in the art, including by classical breeding, protoplast fusion, nuclear transfer and chromosome transfer. Breeding approaches and techniques are known in the art, and are set forth in, for example, J. R.

30  Welsh, *Fundamentals of Plant Genetics and Breeding* (John Wiley and Sons, New York, (1981)); *Crop Breeding* (D. R. Wood, ed., American Society of Agronomy, Madison, Wisconsin, (1983)); O. Mayo, *The Theory of Plant Breeding, Second Edition* (Clarendon Press, Oxford, England (1987)); and Wricke and Weber, *Quantitative Genetics and Selection Plant Breeding* (Walter de Gruyter and Co., Berlin (1986)). Using these and other techniques

35  in the art, transgenic plants and inbred lines obtained according to the present invention may

be used to produce commercially valuable hybrid plants and crops (*e.g.*, hybrid squash and sugarbeets), which hybrids are also an aspect of the present invention.

The following Examples are provided to illustrate the present invention, and should not be
5      construed as limiting thereof.

## EXAMPLE 1: TRANSFORMATION OF SQUASH

### Seed sterilization and germination

Mature embryos of squash (*Cucurbita pepo.* L.) are carefully removed from dry seeds using a
10     pair of pliers.  They are then placed in 50-ml disposable centrifuge tubes and surface-
sterilized in 15% Clorox® solution (V/V) for 15 min.  During sterilization, the tubes are placed
on a rotator and agitated at 200 rpm.  After rinsing three times with sterile water, the embryos
are soaked in sterile water for an additional 20 min.  The tubes are shaken by hand from time
to time to remove the membrane that encloses the embryos.  After sterilization, mature
15     embryos are germinated on a MS-based (Murashige and Skoog, 1962) medium
supplemented with 4.0 mg/L -BA and grown in a culture room at 23-26°C in darkness.

### Preparation of explants

*Cotyledonary petioles.*   Three days after germination, the hypocotyls and two-thirds of the
cotyledons are removed from the germinating seedlings.  The cotyledonary petiole explants,
20     which contain the cotyledonary nodes, shoot tips and the remaining of the cotyledons, are
then transferred to a shoot multiplication (SM) medium and grown at 25°C under light.  The
SM medium contains MS salts, B5 vitamins, 2 - 4 mg/L BA- and is solidified with about 4 g/L
(or about 0.4% w/v) Phytagel™ .

Three to ten days after cultivation on the SM medium, excessive tissues of the elongated
25     hypocotyls and cotyledons are carefully removed and the cotyledonary petioles used for
transformation are separated from the shoot tip region.  The cotyledonary petioles can also
be further cultured on the SM medium for an additional three to fourteen days before use in
transformation.

*Shoot tips.* The apical or axillary meristems containing shoot primodia, leaf primodia, young
30     leaves and a portion of hypocotyls are excised from germinating seedlings or plantlets.  They
are maintained on SM medium, grown under light at 25 °C and sub-cultured biweekly. The
shoot tip explants are ready for transformation after the first subculture.  They can also be
used as explants for transformation after several passage of subcultures.

## _Agrobacterium_ strains and plasmids

_Agrobacterium tumefaciens_ strains such as EHA101, LBA4404, GV3101 and K6 (ATCC# 55964) are used for gene delivery with EHA101 (Hood et al, 1986. J Bacteriology 168: 1291-1301) as the preferred strain. The plasmid used for transformation, pNOV2105, contains the coding sequence for phosphomannose isomerase (PMI), which is driven by a modified _Smas_ gene promoter (Ni et al. (1995) Plant J. 7: 661-676). _Smas_ (mannopine synthase) promoter is derived from _Agrobacterium tumefaciens_ and is enhanced by multiplying the _ocs_ enhancer region. The gene is terminated with the nos 3' region. It also contains an _Escherichia coli uidA_ gene that encodes β-glucuronidase (GUS, Jefferson et al (1986) _PROC. NATL. ACAD. SCI. USA_ 83: 8447-8451). A _gus_ intron (Vancanneyt et al. (1990) Mol. Gen. Genet 220 (2):245-250) has been placed inside the GUS coding sequence. The gene is driven by a Smas promoter and terminated with the nos 3' region. Agrobacteria used for transformation are maintained on a Bacto agar-solidified YP medium (5 g/L yeast extract, 10 g/L peptone and 5 g/L NaCl. pH 6.8) which contains appropriate antibiotics (50 mg/L kanamycin, 100 mg/L spectinomycin, and 100 mg/L carbenicillin). The bacteria are kept at 28°C.

## Inoculation and co-cultivation

Three methods are adopted for inoculating agrobacteria to the explants.

_Method 1._ Two to three day old _A. tumefaciens_ colonies are collected from the Bacto™ agar-solidified bacteria plates using an inoculating loop. Right before or during inoculating _Agrobacteria_ to the explants, the meristematic region of cotyledon petioles or meristematic cultures of apical and axillary shoot tips are repeatedly surface-wounded either by a surgical blade or a needle. Bacteria inoculation is carried out by applying _Agrobacteria_ on the surface of the target regions prior to wounding, or the _Agrobacteria_ are "pasted" to the blade or the needle prior to the wounding process. Following wounding, a liquid _Agrobacterium_ induction medium (_e.g._, an AII medium, which comprises SM medium plus 200-600 mg/L acetosyringone, about 1μl to 6 μl depending on the size of the explants) is immediately applied to the wound area in amount of from about one to about six μL, depending on the size of the explants. Excessive _Agrobacteria_ and AII medium on the surface of the wounded tissue are removed by a sterile filter paper. After being air-dried for a short period of time, the infected explants are transferred to a co-cultivation medium (for example, ACC medium, which comprises AII medium in addition to about 20 g/L sucrose and 30 g/L glucose) and incubated at 22°C in darkness for two to four days.

_Method 2._ Two to three day old _A. tumefaciens_ aggregates are collected from YP plates and mixed with 25 –50 μl of AII medium. The bacteria are left at room temperature for 30 min before inoculation. Bacterial infection is carried out by applying a small amount of

-24-

the induced *A. tumefaciens* mixture to the meristematic region of cotyledon petioles, or to the meristematic cultures from apical and axillary shoot tips prior to wounding. Alternatively, bacterial infection is carried by wounding the target region with a blade or the needle coated with *Agrobacterium* aggregates. After being air-dried for a short period of time, the infected

5      explants are transferred to ACC co-cultivation medium.

*Method 3.* *A. tumefaciens* suspensions used for infection are prepared by either suspending two to three day old bacteria aggregates in AII medium on the day of infection, or by growing the bacteria overnight in YP liquid medium and then re-suspending the bacteria in AII medium prior to infection. In the former case, the suspensions are agitated on a slow

10     motion shaker or shaken gently by hand for 0.5 – three hours before use. The final concentration of *A. tumefaciens* is adjusted to $0.2 – 2.0 \times 10^9$ cfu (colony forming units). For infection, the explants are submerged in the *A. tumefaciens* suspension and the target regions are wounded either with a blade or with a needle. After infection, excessive liquid on the explants is blotted off with a filter paper and the explants are air-dried for 30 to 60 min

15     until the liquid on the surface cannot be seen. The infected explants are then transferred to ACC medium for co-cultivation.

**Shoot multiplication and selection**

After co-cultivation at ACC medium for two to four days, the infected explants are transferred to a shoot multiplication/selection medium (MSS) and grown at 25°C under light for two

20     weeks. Subsequent subcultures and selection are carried out at two-week intervals. With each subculture, mannose-resistant green shoot clumps are selected, cut into smaller pieces and transferred to fresh MSS medium. The MSS medium consists of SM medium and antibiotics, either with or without mannose. The concentration of mannose in MSS medium is gradually increased from 0 g/L to 2 g/L, 3 g/l and 4 g/L through the subsequent selection

25     while the concentration of sucrose is decreased from 30 g/L to 25g/L and then to 20 g/L.

**Plant regeneration**

Shoot clumps growing on mannose are maintained and new clones are made on MSS medium that contains two to four g/L mannose and 0.5 – four mg/L of 6-benzyl-aminopurine (BA or 6-BAP). To promote shoot elongation, shoot clumps are grown on MSS medium with

30     or without 0.5 mg/L BA. Shoots are rooted in MSS medium without BA or MSS medium containing 1 mg/L indole-3-butyric acid (IBA). Rooted shoots are transferred to soil and grown to maturity in the greenhouse. Transgenic plants are self-pollinated and/or cross-pollinated with non-transgenic plants to produce T1 progenies. The T1 transgenic progenies are harvested for analysis and are used for producing T2 transgenic progenies.

35

## Plant analysis

*GUS histochemical assay.* The tissues or organs from transgenic plants and non-transgenic control plants are incubated in a GUS substrate at 37°C overnight according to the method of Jefferson (*Plant Mol. Bio. Rep.* 5, 387-405 (1987)). To help visualize GUS expression in leaf tissues, chlorophyll is extracted from the tissues using 70% ethanol, if needed. Transgenic progeny containing the *gus* gene are screened by performing a GUS histochemical assay on the roots and immature embryos of the progeny. Segregation analysis of the transgene in progeny is performed using a Chi square test.

*ELISA assay.* Leaf samples are collected from the transgenic and non-transgenic plants, and an ELISA plus/minus assay is performed to confirm the presence of the PMI enzyme.

*Integration of the transgene.* Total DNA is isolated from transgenic plants and non-transgenic control plants. The integration of transgene in the plant genome is confirmed by Southern blot analysis. Results from the transformation with EHA 101 (pNOV2105) are summarized in **Table 1**.

**Table 1**

| Experiment | No. of Explants | PMI+ and GUS+ | % Expression (GUS+ / No. Explants) |
|---|---|---|---|
| 1 | 200 | 2 | 1 |
| 2 | 50 | 1 | 2 |
| 3 | 200 | 3 | 1.5 |
| 5 | 150 | 4 | 2.7 |
| 6 | 200 | 21 | 10.5 |
| 7 | 100 | 4 | 4 |

The transformation efficiency in squash using the multiple shoot transformation approach described in this invention ranged from 1 % to 10.5%. Strong GUS activity was observed in the leaves, leaf petioles, roots, stems, shoots, anthers, corolla, calyx, filaments, nectars, pollen grain, stigmas, styles, ovules, epidermis of ovary and immature embryos of the transgenic squash plants. Southern blot analysis confirmed the integration of *pmi* gene in the genome of the transgenic plants. Inheritance of the transgene (GUS) in the progeny was confirmed by a GUS histochemical assay. Chi square analysis was performed to check the segregation of transgene (GUS) in the progeny. The results showed that the GUS gene segregated at a 3:1 ratio among the progenies (T1's) that were self-pollinated and at a 1:1 ratio among the progenies that were cross-pollinated with non-transgenic plants. Six T2 progenies from 1 self-pollinated T1 plant were selected for further analysis. A total of 136 embryos were collected from those 6 (six) T2 plants and analyzed for the presence of GUS

and PMI genes.  The results were all positive, confirming the recovery of a homozygous event.

## EXAMPLE 2: TRANSFORMATION OF WATERMELON

Mature seeds of watermelon (*Citrullus lanatus* (Thumb.) Mansf.) are germinated and  multiple shoot cultures are prepared as described in Example 1.  The transformation steps and media used for watermelon transformation, including *Agrobacteria* infection, co-cultivation,selection plant regeneration, are performed as described in Example 1.  Results from the transformation with *Agrobacterium tumefaciens* EHA101 harboring pNOV2105 are shown in **Table 2**.  Strong GUS activities were observed in most of the vegetative and reproductive organs and tissues, as described in Example 1. Expression of GUS activities was also observed in the progeny from the self-pollinated transgenic watermelon plants.

**Table 2**

| Experiment | No. of Explants | Transgene | PMI+/GUS+ | % Expression |
|---|---|---|---|---|
| 1 | 50 | *uidA* | 3 | 6 |

## EXAMPLE 3: TRANSFORMATION OF MELON

The seed coat from melon (*Cucumis melo* L) seeds is removed using a pair of forceps and the seeds are sterilized for 1 min. in 70% ethanol followed by 10 min. in 1% hydrochloride. The seeds are rinsed three to four times with sterile water and sowed on a half-strength hormone-free MS medium, at 10 seeds per plate. The seeds are germinated at 22-24°C for 16 hrs at 3000-4000 lux and for eight hours in darkness.  After seven days, the cotyledon and petioles are separated from the seedlings and transferred them to MS30 medium (MS salts, 30 g/L sucrose). One day before transformation, an *A. tumefaciens* culture is started in a universal tube by transferring 50µl of a stock solution into 5 ml LB medium containing antibiotics.  The culture is incubated at 28°C at 150 rpm for a minimum of 17 hours. On the day of transformation, 1.2 to 1.5 ml of the overnight-grown *Agrobacterium* culture is added to the plates containing the cotyledons (final *Agrobacterium* concentration: $2x10^8$ cfu/ml). Small incisions (wounding) are made in the cotyledon petioles at the site of the meristem.  The plates with *Agrobacterium* + explants are transferred in a vacuum dessicator. Vacuum is applied by a vacuum pump for 1 minute. The vacuum is held for 15 minutes. After 15 minutes the vacuum is released slowly. The *Agrobacterium* suspension is soaked off the

-27-

plate. The explants are washed with liquid MS30 by adding MS30 to the plates. The MS30 is soaked from the plates and the explants are dried on sterile filter paper. The explants are placed on BM4.5 + 0.5 mg/l BAP (BM4.5B), at 20-30 explants per plate. The plates are incubated at 21-23°C for 16 hours at 1500-2000 lux and then for eight hours of darkness for

5   five days. After five days of co-cultivation, the explants are transferred to a selection medium containing mannose, 0.5 mg/L BA, 0.125 mg/L ancymidol and 1.5 mg/L $AgNO_3$, at ten explants per plate. The plates are placed at 24°C for 16 hrs at 2000-2500 lux and then placed in darkness for eight hrs, and transferred every two to 2.5 weeks to fresh medium. Shoots of 0.5 mm and larger are transferred to BMM-c medium that contains MS salts, 0.1

10  mg/l NAA, 3 mg/l AgNO3 and 200 mg/l cefotaxime and 100 mg/l vancomycine. The shoots are transferred every two to three weeks to fresh BMM-c medium.


## EXAMPLE 4: TRANSFORMATION OF SUNFLOWER

Sunflower (*Helianthus annuus* L.) seeds are germinated as described in Example 1. Multiple

15  shoot cultures are prepared from the original shoot tip or from the immature inflorescence using the media as described in Example 1. Immature inflorescences are taken from the greenhouse-grown young plants or from *in vitro* cultured shoot tips. The multiple shoot cultures are transformed with *Agrobacterium tumefaciens* EHA 101 harboring pNOV2105. Strong GUS activity is observed in multiple shoot cultures four weeks after infection.

20  Transgenic plants are recovered following the selection and shoot multiplication steps as described in Example 1. The results are summarized in **Table 3**.


**Table 3**

25

| Experiment | No. of Explants | Transgene | PMI+/GUS+ | % Expression |
|------------|-----------------|-----------|-----------|--------------|
| 1          | 7               | *uidA*    | 7         | 100          |
| 2          | 10              | *uidA*    | 10        | 100          |

30


## EXAMPLE 5: TRANSFORMATION OF SUGARBEET

### Seed sterilization and germination

35  Seeds of sugarbeet (*Beta vulgaris* L.) are scarified using concentrated sulfuric acid for 10 minutes. After thorough rinsing of the seeds to remove residual sulfuric acid, seeds are soaked overnight in water. Surface sterilization is performed the next day using 20% Clorox® (commercially available) for 15 minutes while shaking. Afterwards, seeds are rinsed 5 times

-28-

with sterile water, then allowed to air dry for 6 hours prior to plating onto seed germination medium (GM) under aseptic culture. In one example, the GM comprised contains Murashige and Skoog (MS) salts (Sigma Chemicals, St. Louis, Mo.) with about 30 g/L sucrose. $B_5$ vitamins (also obtained from Sigma Chemicals), myo-inositol (100 mg/L), pantothenic acid (1

5  mg/L) and Phytagel® at 3.6 g/L were also included in the GM, as were plant growth regulators with cytokinin-like function. Cytokinin levels are generally within a typical range of 0.5 mg/L to 5 mg/L, and usually between 1.0 and 2.0 mg/L. For sugar beet seeds, the auxin inhibitor TIBA is also added.

**Excision and initiation of shoot meristematic cultures**

10  Shoot tips of 7-14 day old seedlings are excised and plated onto shoot multiplication medium (SMM). In this case, the SMM comprised Murashige and Skoog salts, 30 g/L sucrose, $B_5$ vitamins and Phytagel 8 g/L. In addition, the SMM contained at least one cytokinin growth regulator such as BA, kinetin, 2-ip or zeatin, generally within a concentration range of about 1 to 10 mg/L and usually within a concentration range of 2-6 mg/L. Additional growth

15  regulators such as GA and those with auxin-like function such as IAA, NAA and 2,4-D were also added.

The shoot tips consisted of both apical and axillary shoot meristematic regions, leaf primordia, 3-4 mm of hypocotyl and the cotyledonary leaves which are cut off to reduce further elongation. Every 7-10 days following plating, target explants were subcultured to

20  fresh SMM after removing any new elongated leaf material. Multiple shoot target explants are typically cultured under low light intensity (10-30 μEinsteins) for 12 to 16 hour day-lengths at 20°C. After 4 to 6 weeks the multiple shoot cultures resemble compact rosettes and are ready for transformation.

**Inoculation and incubation of multiple shoot culture**

25  *Agrobacterium tumefaciens* mediated transformation is utilized for the transformation of the multiple shoot culture. The *A. tumefaciens* strain EHA101 with plasmid pAD1289 and pNOV2105 (containing the PMI selectable marker gene and the GUS scorable marker gene) is grown on solid culture medium consisting of 5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl and 15 g/L Bactoagar™ for 2-3 days at 28°C. The plasmid pNOV1508 contains *pmi*

30  gene, which is driven by the modified SMas promoter and terminated with the nos 3' region. It also contains a double-mutated *Arabidopsis* PPO gene (see U.S. patent 6,084,155), which is driven by the *Arabidopsis* UBQ3 (intron) promoter (*see* Norris et al., *Plant Molecular Biology* 21,895-906 (1993)) and terminated with the 35S 3' region. One day prior to transformation the multiple shoot culture is prepared for inoculation by removing any

35  remaining elongated leaf material.

-29-

To begin inoculation, single colonies of A. tumefaciens are collected together on the original YP culture plate using a sterile loop. For the actual inoculation of each target explant, a sterile scapel blade is dipped into the collected A. tumefaciens colonies and used to make cuts in the apical and axillary meristem regions of each target. Immediately following this

5 inoculation step, about 4 to 6 μl of MSMG Induction Medium (MS salts, 2 g/L Glucose, MES, and 200μM acetosyringone) is applied to the wounded surface of each target in some experiments. An effort is made to cut through the center of as many meristematic zones as possible in order to direct gene delivery to shoot meristem producing cells. Ten to twenty target cultures are typically treated in sequence and then allowed to air dry under sterile

10 conditions in a laminar flow hood for 10 minutes. Following the air drying treatment, treated target explants are moved to MSCC co-cultivation medium (MS salts, B5 vitamins, 2 mg/L BA, 30 g/L sucrose, 200μM acetosyringone). The treated explants are then incubated on MSCC medium for 2-3 days at 22°C with continuous dark culture.

**Target culture and selection**

15 Following inoculation and co-cultivation, the multiple shoot explants are transferred to fresh SMM medium with 2 mg/L BA and appropriate antibiotics for a minimum of two days before applying mannose selection pressure. Transformed tissues are selected on gradually increasing amounts of mannose (2 g/L – 20 g/L) and decreasing amounts of sucrose (26 g/L – 0 g/L) following transformation. Mannose selection levels are increased in a stepwise

20 manner, from 2 g/L mannose + 26 g/L sucrose to 3 g/L mannose + 24 g/L sucrose, then 4 g/L mannose + 22 g/L sucrose, followed by 5 g/L mannose + 20 g/L sucrose and 6 g/L mannose + 18 g/L sucrose. The multiple shoot cultures continue to grow in size and are carefully divided at each sub-culturing to promote adequate selection pressure. BA levels are maintained at 2 mg/L for typically 16-20 weeks following transformation to continue shoot

25 meristem multiplication under selection. Following this period, the BA level is reduced to 0.25 mg/L for 4-6 weeks and then eliminated to promote shoot elongation. Areas of surviving transformed tissue are continually removed from dying untransformed sections of the original target explant and surviving sections are again carefully divided to promote stringent selection. Selection and shoot regeneration typically progress over a time period of from 10

30 to about 30 weeks. As young shoots emerg they are separated and isolated under selection for the most efficient selection of transformed shoots.

**Elongation of transformed shoots**

Once the young shoots reach approximately 0.5-1.5 cm, they are transferred to GA7's containing shoot elongation medium (elongation of developing shoots is enhanced by

35 reduction of cytokinin levels) with or without mannose selection as described above. The

shoot elongation medium contains MS salts, B₅ vitamins, 30% sucrose and Phytagel™
(7.5g/l). Low levels of cytokinin are incorporated in the elongation medium, within a typical
range of 0.1 to 1.0mg/l. The optimal cytokinin application for sugar beet is 0.5 mg/L kinetin.

**Regeneration of transformed plants**

Transformed shoots are cloned on MS-based cloning medium plus mannose at 5-20g/L.
Multiple shoots from one original transgenic shoot are sometimes desirable, and for this
reason a combination of cytokinin and auxin in the basal MS medium was used to induce
cloning. Low levels of both growth regulators typically range from 0.1mg/L to 0.5mg/L. For
sugar beet, MS salts and 30g/L sucrose with 0.2mg/L kinetin, 0.1mg/L NAA, and Phytagel™
7g/L is used.

Single shoots or clones are successfully rooted when transferred to a rooting medium
containing MS basal medium supplemented with one or two auxins such as IBA or NAA at
0.5mg/L to 5 mg/L. In one example, the rooting medium contains 5mg/L IBA, 0.5mg/L NAA
and about 5-20 g/L mannose. Phytagel™ at 5g/L was found to be an appropriate gelling
agent to support root development.

**Transformation results**

High efficiency transformation was achieved in sugar beet using multiple shoot cultures as
explants

Results from sugar beet multiple shoot cultures transformed with *Agrobacterium tumefaciens*
containing pNOV2105 are given in Table 4. GUS expression from the transformed sugar beet
plants was detected using a histo-chemical assay. Expression of the PMI gene was
determined using a PMI ELISA +/- assay.

**Table 4**

| Experiment | Number of Explants | GUS+ | GUS+ or PMI+ | % Expression (sum of GUS+ or PMI+ / Number of Explants) |
|---|---|---|---|---|
| 1 | 20 | 7 | 10 | 50 |
| 2 | 28 | 16 | 16 | 57 |
| 3 | 31 | 14 | 18 | 58 |
| 4 | 21 | 7 | 7 | 33 |
| 5 | 44 | 7 | 17 | 39 |
| 6 | 41 | 17 | 22 | 54 |
| 7 | 31 | 15 | 15 | 48 |
| 8 | 50 | 20 | 23 | 46 |

In one experiment, where sugar beet multiple shoot cultures were transformed with
*Agrobacterium tumefaciens* containing pNOV1508 (a plasmid that contains the PMI

selectable marker gene and the mutated Arabidopsis thaliana PPO gene), 18 out of 34 explants produced PMI + plants. Further analysis of those PMI+ transgenic sugar beet plants by Northern analysis showed that 7 of them also expressed the mutated *Arabidopsis thaliana* PPO gene. Those PPO expressing plants were sprayed with a PPO inhibitor

5    herbicide at a field-applicable level and several of them showed dramatically high tolerance to the herbicide. Two transgenic PPO events were selected for T1 seed production. These events were confirmed to be transgenic by PMI ELISA and *Southern* analysis *for the PMI* gene. Each was shown to contain multiple copy integrations of the PMI gene. The rooted T0 plants were transferred to soil and grown for 5-6 weeks at greenhouse conditions of 21ºC,

10   60% humidity, 16hr daylength/8hr night. After observing normal phenotypic plant development the T0 plants were transferred to vernalization conditions (4ºC, 16hr day/8hr night) for 16 weeks then acclimated to 14ºC for 2 weeks. Following acclimization the transgenics were returned to greenhouse conditions where normal flower development was observed. The transgenics were self-pollinated and cross-pollinated with non-transgenic

15   plants for seed production. T1 seed was successfully produced, then harvested and sown in soil for germination. T1 seedlings were verified to contain the PMI gene by PMI ELISA analysis. Co-integration of the PMI and PPO transgenes were confirmed by Southern on five T1 plants for each event.

**Elimination of chimeras**

20   Additional steps are taken to reduce the potential of generating transformed plants that are chimeric (partially transgenic). One step is to extend the period of high cytokinin treatment (2 mg/L) to eight to sixteen weeks to continue to stimulate the shoot meristem multiplication culture response under selective pressure. Another step is to increase the stringency of mannose selection (for instance, increase mannose stepwise from 5 g/L to 10 g/L during

25   selection). In one experiment where sugar beet multiple shoot cultures were transformed with EHA101 containing pNOV2105, 35 out of 36 (97%) explants that are analyzed for GUS expression produced completely transgenic shoots (see Table 5 below).

**Table 5**

| Number of Targets | 45 |
|---|---|
| Initial Selection / Weeks | 5 g/L mannose +20 g/L sucrose; 2 weeks |
| Subsequent Selection | 6 g/L mannose + 18 g/L sucrose; 2 weeks<br>7 g/L mannose + 16 g/L sucrose; 2 weeks<br>8 g/L mannose + 14 g/L sucrose; 2 weeks<br>10 g/L mannose + 10 g/L sucrose;4 weeks |

| 2 mg/L BA Treatment | first 8 weeks |
|---|---|
| 0.25 mg/L BA treatment | subsequent 4 weeks |
| Number of targets histochemically assayed (GUS assay) | 36 |
| Number of targets and percentage of targets with fully transgenic shoots (*i.e.* not chimeric) | 35 (97%) |

## EXAMPLE 6

### PLASTID TRANSFORMATION USING MERISTEMATIC CULTURES DERIVED FROM SHOOT TIPS, LEAVES OR OTHER PLANT TISSUES

#### Construction of sugar beet plastid transformation vectors

The *trnV* and *rps12/7* intergenic region of the *Beta vulgaris* plastid genome is amplified and modified for insertion of chimeric genes. A 1559 bp region (positions 1 to 1560, GenBank accession number AB032426) is PCR amplified from the sugar beet plastid genome and a *Pst*I site is inserted at position 525. A first PCR amplification of 536 bp is performed from total DNA of *B. vulgaris* with the following pair of primers: Bv16-MluI (5'TTC TTA CGC GTT ACT CAC CCG 3', SEQ ID NO:1) having a natural *Mlu*I restriction site (underlined), and Bv16S-PstI (5' AAA CTG CAG AAA GAA GTC CCG GCT CCA AGT 3', SEQ ID NO:2) introducing a *Pst*I site (underlined). A second PCR amplification of 1049 bp was performed with the following pair of primers: Bvrps12-BHI (5' AAA GGA TCC AAA TTG ACG GGT TAG TGT G 3', SEQ ID NO:3) introducing a *Bam*HI restriction site (underlined) and Bvrps12-PstI (5'AAA CTG CAG TCG CAC TAT TAC GGA TAC GAA 3', SEQ ID NO:4) introducing a *Pst*I site (underlined). Both PCR reactions are undertaken with *Pfu* thermostable DNA polymerase in a Perkin Elmer Thermal Cycler 480 according to the manufacturer's recommendations (Perkin Elmer/Roche, Branchburg, NJ) as follows: 5 min 95°C, followed by 35 cycles 1 min 95°C/ 1 min 50°C/1 min 72°C, then 10 min at 72°C. The 536 bp amplification product is digested with *Mlu*I and *Pst*I to generate a 525 bp *Mlu*I/*Pst*I fragment representing the sugar plastid *trnV* flanking sequence. The 1049 bp amplification product is digested with *Pst*I and *Bam*HI to generate a 1034 bp *Bam*HI/*Pst*I fragment representing the sugar beet *rps12/7* flanking sequence.

The tobacco plastid transformation vector pAT238 (PCT WO 00/20612 Novartis, Int pub date 04/13/00) is cut with *Mlu*I and *Bam*HI and the 3.3 kb *Mlu*I/*Bam*HI vector fragment was isolated. This fragment is ligated in a three-way reaction with 525 bp *Mlu*I/*Pst*I fragment and 1034 bp *Bam*HI/*Pst*I to generate the sugar beet transformation vector pEBcpSBeet

-33-

backbone. The resulting plasmid comprises a chimeric tobacco:: sugarbeet::tobacco plastid *trnV* and *rps12/7* intergenic locus, the 1559 bp sugar beet sequence is flanked by 414 bp tobacco plastid sequence on *trnV* side and 39 bp on the *rps12/7* side. A chimeric *aadA* gene, which confers resistance to spectinomycin, driven by the tobacco *psbA* promoter is

5      inserted in the *PstI* site previously created in the sugar beet sequence. The plasmid clone having the *aadA* cassette in the orientation towards the *rps12/7* flanking region (pEBSB1) is used for subsequent sugar beet multiple shoot transformation.


**Sugar beet plastid transformation**

10     Multiple shoot cultures of sugar beet are derived from shoot tips as described in Example 5. The day before bombardment, target explants are cut either into four pieces (cutting from the top to the bottom of the explant) or kept as intact target explants.
       On the day of the bombardment, sugar beet target explants (whole or cut pieces) are transferred to SMM media with 12% sucrose. Explants are placed in the center of the target

15     plate to maximize gene delivery. Cut explants are oriented with the cut surfaces facing upwards to maximize gene delivery to the internal cells of the explant. Sterilized particles are coated with the previously described sugar beet plastid transformation vector pEBSB1. The DNA-particle preparation and bombardment procedures are similar to the protocols described by the manufacturer.

20     After bombardment, the sugar beet explants are placed back in SMM media for 2 days. The sugar beets are then transferred to SMM media with 250mg/l of spectinomycin for selection. Two weeks later, the tissue is transferred to SMM media containing 500mg/l of spectinomycin for continuous selection. After one to two months of selection, green spectinomycin-resistant shoots appear on the white tissues and are analyzed.

25

**Tobacco plastid transformation**

Meristematic cultures are derived from leaf tissues of tobacco, on media containing at least one cytokinin growth regulator such as BAP, in a concentration range of about 0.5 to 5 mg/L, preferably 1 to 2mg/L. Meristematic cultures are also derived from other parts of tobacco

30     plant. Additional growth regulator(s) such as NAA are also included. In addition to plant regulators, the medium contains MS salts, B5 vitamins, 30g/L sucrose and 3g/L agar, with a pH of about 5.8.
       The day of bombardment, meristematic cultures are transferred to fresh media, and placed in the center of the plate to maximize gene delivery. Sterilized particles are coated with the

35     tobacco plastid transformation vector pAt238 (*see* International Application No. PCT WO

-34-

00/20612). The DNA-particle preparation and bombardment procedures are similar to the protocols described by manufacturer.

Cultures are transferred to selection media containing 500mg/L spectinomycin two days after the bombardment. After about 4 weeks of selection, resistant green shoots appear from the white tissues. These primary resistant shoot tissues are subcultured periodically to fresh media containing 500mg/L spectinomycin.

Three months after selection, 17 independent events from two experiments are analyzed by PCR for aadA gene and the plastid integration. 14 events are confirmed containing aadA and having the plasmid integrated into their plastid genome.

## Tomato plastid transformation

Various explants from tomato tissue (such as cotyledon, leaf, shoot tips, and the like) are cultured on media containing at least one cytokinin growth regulator (such as zeatin, BAP) to induce meristematic cultures. The cytokinin concentration ranges from about 0.5 to 5mg/L, preferably from 1 to 2mg/L. Additional growth regulator(s) such as IAA, NAA are also included. In addition to plant regulators, the medium contains MS salts, B5 vitamins, 30g/L sucrose and 7.5g/L Phytagar, pH5.8.

The day of bombardment, meristematic cultures are transferred to fresh media, and placed in the center of the plate to maximize gene delivery. Sterilized particles are coated with plastid DNA, containing tomato or tobacco homologous sequence flanking the selectable marker gene (and the gene of interest). The DNA-particle preparation and bombardment procedures are similar to the protocols described by manufacturer.

Two days after bombardment, cultures are transferred to the selection media containing 500mg/L spectinomycin. After several weeks of the selection, resistant green shoots will appear from the white tissues. The shoots obtained continue to grow on spectinomycin selection media and contain transformed plastids.

The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

**THAT WHICH IS CLAIMED IS:**

1. A method of producing a transformed dicotyledonous plant, comprising:

(a) culturing a tissue of a dicotyledonous plant recalcitrant for transformation on a culture medium to produce a multiple shoot culture from the tissue;

(b) introducing a nucleic acid into a cell of the multiple shoot culture, thereby producing a transformed cell comprising the nucleic acid; and

(c) regenerating a transformed plant from the transformed cell.

2. The method according to Claim 1, wherein the tissue is meristematic tissue.

3. The method according to Claim 2, wherein the meristematic tissue is excised from a shoot apex, an axillary bud or a floral meristem.

4. The method according to Claim 1, wherein the tissue is callus tissue.

5. The method of Claim 1 wherein the plant is a member of the *Cucurbitaceae* family or of the *Chenopodiacea* family.

6. The method of Claim 1 wherein the plant is selected from the group consisting of sugar beet, sunflower, soybean, cotton, melons, watermelon, squash, *Brassica* and pepper.

7. The method of Claim 1 wherein the plant is sugar beet, squash, melon or watermelon.

8. The method of Claim 1 wherein the tissue is excised from the shoot tip of a seedling of the plant.

9. The method of Claim 1, wherein the culture medium comprises at least one plant growth regulator.

10. The method of Claim 9, wherein the at least one plant growth regulator is a cytokinin.

11. The method of Claim 9, wherein the concentration of growth regulator in the culture medium is between about 0.01 mg/L to about 25 mg/L.

12.  The method of Claim 1, wherein the nucleic acid is introduced into the cell by microparticle bombardment or using a bacterium belonging to the genus *Agrobacterium*.

13.  The method of Claim 1, wherein the nucleic acid comprises a nucleic acid that is heterologous to the dicotyledonous plant.

14.  The method of Claim 1, wherein the nucleic acid comprises a gene that encodes a polypeptide having PPO activity.

15.  The method of Claim 1, wherein the nucleic acid comprises a gene that encodes a polypeptide having phosphomannose isomerase (PMI) activity.

16.  The method of Claim 1, wherein the nucleic acid comprises a gene that encodes a polypeptide having xylose isomerase (xylA) activity.

17.  The method of Claim 1, wherein the nucleic acid comprises a gene that encodes a polypeptide having GUS activity.

18.  The method of Claim 1, wherein step (c) comprises:
selecting a multiple shoot culture comprising a transformed cell;
growing the multiple shoot culture under conditions that promote shoot elongation to produce at least one transformed shoot; and then
growing the at least one transformed shoot into a mature transformed plant.

19.  The method of Claim 19 wherein the at least one transformed shoot grows into a mature transformed plant after growing the at least one transformed shoot on a medium that promotes root formation.

20.  The method of Claim 1, wherein the step (c) comprises:
selecting a multiple shoot culture comprising a transformed cell;
growing the multiple shoot culture under conditions that promote shoot elongation to produce at least one transformed shoot;
cloning the at least one transformed shoot; and
allowing the cloned shoot to mature into a transformed plant.

21. The method of Claim 20 wherein the cloned shoot grows into a mature transformed plant after growing the cloned shoot on a medium that promotes root formation.

22. A transformed plant cell produced by the method of any one of claims 1-21.

23. A multiple shoot culture produced by the method of any one of claims 1-21.

24. A transformed plant produced by the method of any one of claims 1-21.

25. The transformed plant according to Claim 24, wherein the plant is a squash plant that expressed a polypeptide having PMI activity.

26. The transformed plant according to Claim 24, wherein the plant is a melon plant that expressed a polypeptide having PMI activity.

27. The transformed plant according to Claim 24, wherein the plant is a watermelon plant that expresses a polypeptide having PMI activity.

28. The transformed plant according to Claim 24, wherein the plant is a sugar beet plant that expresses a polypeptide having PPO activity.

29. A seed produced by the transformed plant of Claim 24, wherein the seed comprises the nucleic acid transformed into the multiple shoot culture.

30. A method of producing a plant comprising a transformed plastid genome, comprising:

(a) culturing a tissue of a plant on a culture medium to produce a multiple shoot culture from the tissue;

(b) introducing a nucleic acid into a plastid genome of a cell of the multiple shoot culture, thereby producing a transformed plastid genome of said cell comprising the nucleic acid; and

(c) regenerating a transformed plant from the transformed cell.

31. The method according to Claim 30, wherein the tissue is meristematic tissue.

-38-

32. The method according to Claim 30, wherein the meristematic tissue is excised or derived from a shoot apex, an axillary bud or a floral meristem or leaf tissue.

33. The method according to Claim 30, wherein the tissue is callus tissue.

34. The method of Claim 30 wherein the plant is a dicotyledonous plant.

35. The method of Claim 30 wherein the dicotyledonous plant is sugar beet, tobacco or tomato.

36. The method of Claim 30 wherein the tissue is excised from the shoot tip of a seedling of the plant.

37. The method of Claim 30, wherein the culture medium comprises at least one plant growth regulator.

38. The method of Claim 37, wherein the at least one plant growth regulator is a cytokinin.

39. The method of Claim 37, wherein the concentration of growth regulator in the culture medium is between about 0.01 mg/L to about 25 mg/L.

40. The method of Claim 30, wherein the nucleic acid is introduced into the cell by microparticle bombardment.

41. The method of Claim 30, wherein the nucleic acid comprises a nucleic acid that is heterologous to the dicotyledonous plant.

42. The method of Claim 30, wherein step (c) comprises:
selecting a multiple shoot culture comprising a transformed cell;
growing the multiple shoot culture under conditions that promote shoot elongation to produce at least one transformed shoot; and then
growing the at least one transformed shoot into a mature transformed plant.

43. The method of Claim 42 wherein the at least one transformed shoot grows into a mature transformed plant after growing the at least one transformed shoot on a medium that promotes root formation.

44. The method of Claim 30, wherein the step (c) comprises:

selecting a multiple shoot culture comprising a transformed cell;

growing the multiple shoot culture under conditions that promote shoot elongation to produce at least one transformed shoot;

cloning the at least one transformed shoot; and

allowing the cloned shoot to mature into a transformed plant.

45. The method of Claim 44 wherein the cloned shoot grows into a mature transformed plant after growing the cloned shoot on a medium that promotes root formation.

46. A transformed plastid genome produced by the method of any one of claims 30-45.

47. A plastid comprising a transformed plastid genome of claim 46.

48. A transformed plant cell comprising a plastid genome of claim 46.

49. A transformed plant comprising a plant cell of claim 48.

50. A seed produced by a transformed plant of claim 49, wherein the seed comprises the nucleic acid transformed into the multiple shoot culture.

SEQUENCE LISTING

<110> Syngenta Participations AG

<120> METHODS FOR STABLE TRANSFORMATION OF PLANTS

<130> S-31514A

<140>
<141>

<150> US 60/224,934
<151> 2000-08-11

<160> 4

<170> PatentIn Ver. 2.1

<210> 1
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      oligonucleotide

<400> 1
ttcttacgcg ttactcaccc g                                                    21


<210> 2
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      oligonucleotide

<400> 2
aaactgcaga aagaagtccc ggctccaagt                                           30


<210> 3
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      oligonucleotide

<400> 3
aaaggatcca aattgacggg ttagtgtg                                             28


<210> 4
<211> 30
<212> DNA

1

```
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      oligonucleotide

<400> 4
aaactgcagt cgcactatta cggatacgaa                                    30
```

Case S-31514A

1

2

(54) Title: METHODS FOR STABLE TRANSFORMATION OF PLANTS

(57) Abstract: Multiple shoot structures are induced from plant tissues (e.g., shoot apices or axillary buds on an artificial medium) to produce multiple shoot cultures. These multi-shoot cultures are then transformed by known transformation methods. Plants are subsequently regenerated from the transformed cells. Crops that may be efficiently transformed by this method include plants normally recalcitrant to transformation such as sugar beet, sunflower, soybean, cotton, tobacco, tomato, peanuts, melons, watermelon, squash, Brassica, and pepper.

# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 7   C12N15/82    A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | BARWALE U B ET AL: "SCREENING OF GLYCINE-MAX AND GLYCINE-SOJA GENOTYPES FOR MULTIPLE SHOOT FORMATION AT THE COTYLEDONARY NODE" THEORETICAL AND APPLIED GENETICS, vol. 72, no. 3, 1986, pages 423-428, XP001079639 ISSN: 0040-5752 page 424, column 1, last paragraph -page 428, last paragraph; tables --- | 1-29 |
| Y | EP 0 301 749 A (AGRACETUS) 1 February 1989 (1989-02-01) page 3, line 40 - line 57 page 7, line 33 - line 44 --- | 1-29 |
|   | -/-- |   |

[X] Further documents are listed in the continuation of box C.  [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 June 2002 | 0 5. 09. 02 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016 | Burkhardt, P |

Form PCT/ISA/210 (second sheet) (July 1992)

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
| A | BABU P ET AL: "In vitro regeneration and Agrobacterium mediated transformation in gladiolus." JOURNAL OF HORTICULTURAL SCIENCE & BIOTECHNOLOGY, vol. 75, no. 4, July 2000 (2000-07), pages 400-404, XP001079530 ISSN: 1462-0316 abstract; figures page 401, column 2, paragraph 2 -page 402, column 1, paragraph 1 | 1-29 |
| A | HALDRUP A ET AL: "The xylose isomerase gene from Thermoanaerobacterium thermosulfurogenes allows effective selection of transgenic plant cells using D-xyloe as the selection agent" PLANT MOLECULAR BIOLOGY, NIJHOFF/JUNK, THE HAGUE, NL, vol. 37, May 1998 (1998-05), pages 287-296, XP002129151 ISSN: 0167-4412 the whole document | 1-29 |
| A | REED J N ET AL: "The use of phosphomannose isomerase (pmi) as an efficient selectable marker for monocot and dicot transformation." IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY ANIMAL, vol. 36, no. 3 Part 2, March 2000 (2000-03), page 34.A XP001079557 Meeting of the Society for In Vitro Biology World Congress on In Vitro Biology;San Diego, California, USA; June 10-15, 2000 ISSN: 1071-2690 the whole document | 1-29 |
| A | WO 95 34659 A (CIBA GEIGY AG ;WARD ERIC RUSSELL (CH); VOLRATH SANDRA (US)) 21 December 1995 (1995-12-21) the whole document | 1-29 |
| P,X | US 6 140 555 A (REICHERT NANCY A ET AL) 31 October 2000 (2000-10-31) column 10, line 41 -column 12, line 21 column 26, line 38 - line 51 column 15, line 15 - line 67; claims | 1-29 |

# INTERNATIONAL SEARCH REPORT

---

**Box I . Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

---

**Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

    1-29

**Remark on Protest**      ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

---

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1 - 29

   relating to a method for nuclear transfromation of dicot plants through transformation of cells from multiple shoots

2. Claims: 30 - 50

   relating to a method for the production of transplastomic plants through plastid transformation of cells from multiple shoots

# INTERNATIONAL SEARCH REPORT

Information on patent family members

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|---|
| EP 0301749 | A | 01-02-1989 | US | 5015580 A | 14-05-1991 |
| | | | AT | 102251 T | 15-03-1994 |
| | | | AU | 619196 B | 23-01-1992 |
| | | | AU | 2019688 A | 02-02-1989 |
| | | | CN | 1030940 A,B | 08-02-1989 |
| | | | DE | 3888040 D | 07-04-1994 |
| | | | DE | 3888040 T | 07-07-1994 |
| | | | JP | 1080296 A | 27-03-1989 |
| | | | NZ | 225290 A | 26-04-1990 |
| | | | US | 5120657 A | 09-06-1992 |
| | | | US | 6084154 A | 04-07-2000 |
| WO 9534659 | A | 21-12-1995 | AU | 706763 B | 24-06-1999 |
| | | | AU | 2453895 A | 05-01-1996 |
| | | | BG | 101115 A | 31-10-1997 |
| | | | BR | 9508030 A | 16-09-1997 |
| | | | CN | 1309184 A | 22-08-2001 |
| | | | CN | 1150820 A | 28-05-1997 |
| | | | CZ | 9603674 A | 18-03-1998 |
| | | | EP | 0769059 A | 23-04-1997 |
| | | | FI | 964958 A | 11-12-1996 |
| | | | HU | 76353 A | 28-08-1997 |
| | | | JP | 10502524 T | 10-03-1998 |
| | | | PL | 317759 A | 28-04-1997 |
| | | | PL | 183091 B | 31-05-2002 |
| | | | SK | 161096 A | 08-10-1997 |
| | | | US | 6288306 B | 11-09-2001 |
| | | | US | 6177245 B | 23-01-2001 |
| | | | US | 6307129 B | 23-10-2001 |
| | | | US | 6282837 B | 04-09-2001 |
| | | | US | 5767373 A | 16-06-1998 |
| | | | US | 2001016956 A | 23-08-2001 |
| US 6140555 | A | 31-10-2000 | NONE | | |